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Identification of the A_3 adenosine receptor in rat retinal ganglion cells

Mei Zhang,[1,2] Murat T. Budak,[3,4] Wennan Lu,[1] Tejvir S. Khurana,[4,5] Xiulan Zhang,[1,6] Alan M. Laties,[1] Claire H. Mitchell[5]

Departments of [1]Ophthalmology, [3]Cell and Developmental Biology, [5]Physiology, and [4]Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, PA; [2]Eye Institute and Xiamen Eye Center, Xaimen University Medical School, Xiamen, People's Republic of China; [6]Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, People's Republic of China

Purpose: Adenosine can protect retinal ganglion cells from the death that accompanies a general ischemic challenge as well as excitotoxic death. In other tissues, both A_1 and A_3 adenosine receptor subtypes can mediate protection. While a role for the A_1 adenosine receptor in ganglion cell protection has been established, a potential for the A_3 receptor has only recently been proposed. Although the pharmacology is promising, the molecular identity of the responsible receptor is unclear as previous studies were unable to detect message for the A_3 receptor in retinal ganglion cells. We combined laser capture microdisection (LCM) and immunopurification with traditional and real-time PCR to unequivocally demonstrate the presence of the A_3 receptor message in rat retinal ganglion cells.

Methods: Retinal ganglion cells of Long-Evans rat pups were retrograde labeled with aminostilbamidine. Eyeballs were enucleated, embedded, frozen, sectioned, and fluorescent cells in the ganglion cell layer were collected with LCM. Purified ganglion cells were also isolated with a two-step panning procedure. cDNA for the A_3 receptor obtained from the microdissected ganglion cell layer, immunopurified ganglion cells, whole retina and testis was amplified using RT-PCR, confirmed by DNA sequencing and compared with published sequences. A_3 receptor message was also amplified using real-time PCR. Ca^{2+} levels in immunopanned ganglion cells were measured ratiometrically with fura-2.

Results: RNA from immunopurified ganglion cells and from dye-loaded cells in the ganglion cell layer contained message for the A_3 receptor when amplified with either traditional RT-PCR or real-time PCR. The entire encoding region was sequenced and found to be 99% identical to the published code. The sequence closely resembled the consensus form of the gene, with other sequences deviating from this default code. Molecular identification was functionally confirmed in purified ganglion cells as the A_3 receptor agonist Cl-IB-MECA prevented the excessive Ca^{2+} rise triggered by $P2X_7$ agonist BzATP.

Conclusions: Retinal ganglion cells express A_3 adenosine receptor mRNA. Stimulation of this receptor can reduce the Ca^{2+} overload following excessive activation of $P2X_7$ receptors.

Growing evidence indicates that adenosine is an important intracellular mediator in the retina and has considerable potential to protect retinal neurons [1-3]. Changes in retinal adenosine levels occur during ischemic events [4-6], with increased adenosine levels both enhancing the recovery of the electroretinogram (ERG) b-wave after ischemia, and preventing the ischemia-induced thinning of retinal cell layers [1]. Although the A_1, A_{2A}, A_{2B}, and A_3 subtypes of G-protein-coupled surface receptors are all stimulated by adenosine [7-9], protective effects on retinal ganglion cells have been primarily associated with the A_1 receptor. Stimulation with A_1 receptor agonists protects the ischemic retina [1-3], and attenuates the damaging effects of N-methyl-D-aspartate (NMDA) [10-12]. The A_1 and the A_3 receptors are both coupled to the G_i protein and their stimulation can decrease cytoplasmic levels of cAMP [13], although other downstream signaling pathways may differ [14-16]. In both neural and cardiac tissues, stimulation of the A_3 receptor with moderate levels of agonists can produce protective actions which equal or exceed those of A_1 receptor agonists, while excessive receptor stimulation is deleterious [17-19]. In studies of cerebral ischemia, postischemic and chronic preischemic administration of A_3 receptor agonists was neuroprotective, whereas acute administration of agonists during ischemia exacerbated the histological and functional damage [19-23]. These reports suggest that stimulation of the A_3 receptor could augment the protective effects of the A_1 receptor on retinal ganglion cells, if the conditions of agonist delivery were examined with care.

A recent study suggested that stimulation of the A_3 receptor was beneficial to retinal ganglion cells [24]. Agonists for the A_3 receptor prevented the Ca^{2+} rise and cell death which accompanied activation of the $P2X_7$ receptor. While this study implied a neuroprotective role for the A_3 receptor using pharmacologic tools, the molecular identification of the A_3 receptor in retinal ganglion cells has been elusive. An initial study was unable to detect any message for the A_3 receptor in the eye using in situ hybridization [25]. Subsequently, message

Correspondence to: Dr. Claire H. Mitchell, Department of Physiology, University of Pennsylvania, 3700 Hamilton Walk, Philadelphia, PA, 19104-6085; Phone: (215) 573-2176; FAX: (215) 573-5851; email:chm@mail.med.upenn.edu

for the A_3 receptor was identified in the whole retinal tissue of mice with the more sensitive conventional reverse transcription-polymerase chain reaction (RT-PCR) technique [26]. This study indicated expression of the A_3 receptor promoter in a few ganglion cells, but was also unable to directly identify message for the A_3 receptor in ganglion cells using in situ hybridization.

The discovery of the neuroprotective potential of the A_3 receptor on retinal ganglion cells makes the molecular identification of the receptor more imperative. It is possible that moderately low levels of A_3 mRNA precluded detection with the in situ technique, while difficulties in isolating ganglion cells prevented the use of more sensitive amplification tools. In the present study, several approaches were employed to overcome these difficulties and directly demonstrate the presence of mRNA for the A_3 adenosine receptor in rat retinal ganglion cells. Message for the A_3 receptor obtained from retrograde labeled cells in the ganglion cell layer using laser capture microdissection (LCM), and from ganglion cells purified using a two-step immunopanning technique, was amplified using both traditional and real-time PCR. The receptor was cloned and sequenced to verify its identity, while physiologic effects in purified cells supported functional confirmation. Together these results provide direct evidence for the A_3 adenosine receptor in retinal ganglion cells.

METHODS

Retrograde-labeling of retinal ganglion cells: Pups at postnatal day (PD) 3-5 from untimed pregnant Long-Evans rats (Jackson Laboratory Inc., Bar Harbor, ME) were back retrograde labeled by the injection of FluoroGold derivative aminostilbamidine (Molecular Probes, Eugene, OR) based upon protocols described previously [27]. Pups were anesthetized with an intraperitonial injection of 50/5 mg/kg ketamine/xylazine. An incision exposed the skull, while a 1 mm hole drilled through the skull exposed the cortex overlying each superior colliculus. Using a Hamilton syringe affixed to a micromanipulator, a needle was inserted 0.8 mm lateral from the midline and 0.8 mm anterior to Bregma's line and a total of 2.5 μl saturated dye dissolved in 0.9% saline was delivered to each side at a depth of 2 mm and 1 mm. The needle was retracted after a delay of 2 min to allow dye absorption and the wound closed with 2-3 sutures. Preliminary examination of labeled retinal whole mounts confirmed an even distribution of dye, showing a high degree of staining 2 days after injection. Consequently, eyes were enucleated 4-5 days after injection, at PD 7-10. Another three unlabeled pups were used to obtain RNA from the whole retina. Animals were sacrificed by i.p. injection of 50/5 mg/kg ketamine/xylazine followed by an overdose, in accordance with University of Pennsylvania IACUC approved protocols and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Tissue preparation and laser-capture microdissection (LCM): Eyeballs from three Fluorogold labeled pups were quickly enucleated, embedded with OCT tissue embedding medium (Tissue Tek, Sakura, Tokyo, Japan) and snap-frozen in liquid nitrogen. The eyeballs were sectioned transversely into 7-10 μm sections with a cryostat, mounted on Superfrost Plus electrostatically charged slides (Fischer Scientific, Pittsburgh, PA) and stored at -80 °C until use. Histogene LCM frozen-section staining reagents were used for tissue dehydration and staining as described by the manufacturer (Arcturus Engineering, Mountain View, CA). Briefly, sections were fixed and dehydrated sequentially in 75%, 95%, and 100% ethanol followed by 100% xylene. Sections were allowed to air dry and were stored in a desiccator at room temperature until utilized for LCM; care was taken to ensure that LCM was completed within 1 h of the slides being placed in the desiccator. Two to three sections from each sample were stained with hematoxylin and eosin (H&E) to verify the quality of the section. A PixCell II LCM System (Acturus Engineering, Mountain View, CA) was used for LCM. Fluorogold labeled retinal ganglion cells were visualized using fluorescent microscopy. Caps and thermoplastic film were placed above the frozen section. The fluorescent cells with large cell bodies characteristic of ganglion cells were then selectively captured from the retina of each section with the 7.5 μm laser setting. The melted thermoplastic membrane and small region of tissue directly underneath adhering to it were removed by lifting the plastic cap. Approximately 500-1,000 ganglion cells were obtained from each specimen (typically from 6-8 slides containing 24-32 sections), with each specimen from a pup derived from a separate breeding pair.

For the H&E staining, sections were air dried for several minutes, stained with 0.1% Mayer's hematoxylin for 10 min, rinsed in tap water for 5 min, and stained with 0.5% eosin for 30-60 s followed by a further wash. Sections were dehydrated using consecutive exposure to 70%, 95%, and 100% ethanol followed by 100% xylene. Sections were then mounted and examined using brightfield microscopy.

Table 1. Primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')	Position	Length (bp)	Ref.
A3-AB	GGTCTACGATCCTGTCAAGGAC	AGTCCCACCAGAAAGGACACTA	109-749	641	[31]
A3-CD	CATGTCCTG TGTGCTTCTG	GGCTCTTTATCTGTCATGGT	576-1299	726	
A3Q	GGTCCACTGGCCCAT ACACA	CGTAGGTGATTTGCAACCACA	245-367	123	[32]
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC			[31]

A_3U represents primer to detect the upper part of the encoding sequence of A_3 adenosine receptor; A_3L represents primer to detect the lower part of the encoding sequence of A_3 adenosine receptor; A_3Q represents primer for the real-time PCR to detect A_3 adenosine receptor message, while GAPDH represents the control sequences used for real-time PCR reactions.

RNA isolation and linear RNA amplification of microdissected retinal ganglion cells: Five independent total RNA preparations were produced from the five sets of retinal ganglion cells obtained using LCM using the Pico pure RNA isolation kit (Arcturus Engineering). Total RNA was extracted from the captured cells by incubating LCM caps in extraction buffer for 30 min at 42 °C. RNA was purified using preconditioned MiraCol (Arcturus Engineering) purification columns. Eluted RNA was directly used for linear RNA amplification.

Linear T7-based RNA amplification was carried out by using the RiboAmp OA kit as suggested by manufacturer (Arcturus Engineering, Mountain View, CA). Total RNA was incubated with hybrid primers containing oligo dT/T7 RNA polymerase binding site containing primers, the RNA was reverse-transcribed into double-stranded cDNA and this cDNA was purified using MiraCol columns. Next, in vitro transcription was performed using T7 RNA polymerase, and the amplified antisense RNA (aRNA) purified using MiraCol columns. The aRNA was quantified and verified using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Upsala, Sweden). The total aRNA yields from each preparation were 1.8-6.0 μg, and the 260/280 ratio between 1.9-2.1 suggested the sample was of high quality.

Retinal ganglion cell panning: Purification of ganglion cells using the immunopanning procedure was performed as described previously [27] based upon the method of Hartwick et al. [10]. Neonatal rat retinas were dissociated enzymatically for 30 min with 15 U/ml papain, 0.2 mg/ml DL-cysteine and 0.004% DNAse I (Worthington/Cooper, Lakewood, NJ). The cells were washed, centrifuged, resuspended, and filtered through a 20 μm nylon mesh. Cells were incubated with rabbit antimacrophage antibody (1:75, Accurate Chemical, Westbury, NY), then incubated in a 100 mm dish coated with goat anti-rabbit IgG antibody (1:400, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Non-adherent cells were removed to a second petri-dish coated with goat anti-mouse IgM (1:300, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and anti-Thy 1.1 antibody (from hybridoma T11D7e2; American Type Culture Collection, Rockville, MD). After 30 min, non-adherent cells were washed off and the remaining cells were incubated with 0.125% trypsin for 8 min at 37 °C. Digestion was stopped with fetal bovine serum (30%) in neural basal medium and cells were centrifuged and plated on coverslips coated with poly-L-lysine and laminin. Total RNA was extracted from immunopanned cells using the TRIzol LS reagent (Invitrogen, Carlsbad, CA). RNA was purified using bromochloropropane (BCP, Molecular Research Center, Inc., Cincinnati, OH), then re-purified using the RNeasy Micro kit (Qiagen Sciences Inc., Germantown, MD) and eluted with 15 μl of DEPC-treated water. RNA was extracted from five separate preparations of immunopanned cells. Images were taken with a Nikon Eclipse E600 microscope equipped for epifluorescence with an excitation filter of 360±40 nm and with emission >515 nm.

RNA extraction from whole retina and testis: Eyeballs from three PD8-9 Long-Evans pups were enucleated. Retinas were dissected quickly from both eyes under microscope. Testes were used as positive control for A_3 receptor detection. A small part of testis from three male adult rats was dissected quickly. Total RNA preparations were made from each specimen using Trizol reagent as above.

Reverse transcriptase polymerase chain reaction and sequencing: RNA from the immunopanned ganglion cells, microdissected cells of the ganglion cell layer, whole retina and testis was reverse transcribed to cDNA using Superscript II (Invitrogen Carlsbad, CA) reverse transcriptase (RT) following the protocol provided by the manufacturer. Polymerase chain reaction (PCR) was performed using the following conditions: PCR was carried out in a total volume of 25 μl, containing 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM each of the two primers used and 1 U of Taq DNA Polymerase (Promega, Fitchburg, WI). All primers were produced by Invitrogen. Two sets of overlapping primers were used for A_3 adenosine receptor message amplification (Table 1) [28]. Samples were denatured at 94 °C for 45 s (for 641 bp and 724 bp), annealed to primers at 65 °C (for 641 bp) or 56 °C (for 724 bp) for 45 s, and elongated at 72 °C for 60 s (for 641 bp and 724 bp). This was repeated for 30 to 35 cycles, followed by extension at 72 °C for 10 min, using a T gradient 96 instrument (Biometra, Goettingen, Germany). RT-PCR products were separated on a 1.0% agarose gel containing ethidium bromide and visualized under UV light. As the RT-PCR product was present as a single band on agarose gel, the products of RT-PCR were purified with QIAquick PCR purification kit (Qiagen Sciences, Germantown, MD) and underwent sequencing by the dideoxy termination method at the University of Pennsylvania School of Medicine sequencing facilities. Sequence data was analyzed using the MacVector software (version 7.2; Accelrys, Inc., San Diego, CA). The putative phosphorylation sites in the predicted protein sequences were assessed using Scansite [29].

Real-time polymerase chain reaction: Gene expression analysis was performed on an ABI Prism 7900HT machine (Applied Biosystems) using SYBR Green PCR master kit (PE Applied Biosystems, Foster City, CA). PCR primers for A_3 receptor were summarized in Table 1 [30]. PCR primers for real-time amplification are summarized in Table 1 [30]. As an internal control for efficiency of transcription, we simultaneously amplified the housekeeping gene GAPDH [31]. All real-time PCR reactions were done in triplicate. PCR conditions were identical for both genes. Cycle conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 90 s (annealing and extension). At the end of the amplification cycles, samples were heated to 95 °C with a ramp time of 20 min to construct dissociation curves to check that single PCR products were obtained, and verify the lack of product in the absence of template. Real-time PCR product was verified using conventional PCR and sequencing as described above. PCR (35 cycles) conditions were: denaturing at 94 °C for 60 s, annealing to primers at 59 °C for 60 s, and elongating at 72 °C for 60 s.

Intracellular Ca^{2+} measurements: Intracellular Ca^{2+} levels from immunopanned ganglion cells were measured as previously reported [27]. In brief, panned RGCs grown on cover-

slips for 24 h were loaded with 10 μM fura-2 and 2% pLuoronic F-127 (Molecular Probes, Eugene, OR) for 60-90 min at 25 °C, then rinsed for 30 min. Coverslips were mounted on a Nikon Diaphot inverted microscope and visualized with a 40x objective. The field was alternatively excited at 340 nm and 380 nm with a scanning monochromator and the light emitted at >520 nm from a region of interest surrounding individual retinal ganglion cells was imaged with a CCD camera and analyzed (Photon Technologies International, Inc., Lawrenceville, NJ). Cells were perfused with a control solution at the start of Ca^{2+} imaging experiments containing (in mM) 105 NaCl, 4.5 KCl, 2.8 NaHepes, 7.2 Hepes acid, 1.3 $CaCl_2$, 5 glucose, 75 mannitol, pH 7.4. Drugs were dissolved in the control solution. Calibration was performed separately on each cell after the experiment by perfusing cells in the presence of 5 μM ionomycin and control solution (with 1.3 mM

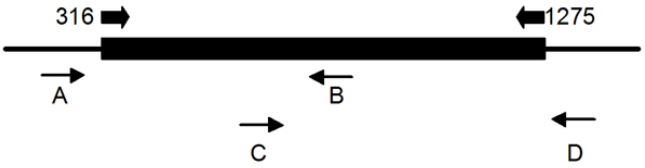


Figure 2. Schematic of rat A_3 receptor cDNA. The encoding region begins at 316 bp and ends at 1275 bp. Primers corresponding to their position on the cDNA are indicated by arrows. Primer A and B yield a product of 641 bp which represents the upper part of the encoding sequence, pair A_3U. Primer C and D yield a product of 724 bp which represents the lower part of the encoding sequence, pair A_3L. There is a 173 bp overlap between two PCR products. See also Table 1.

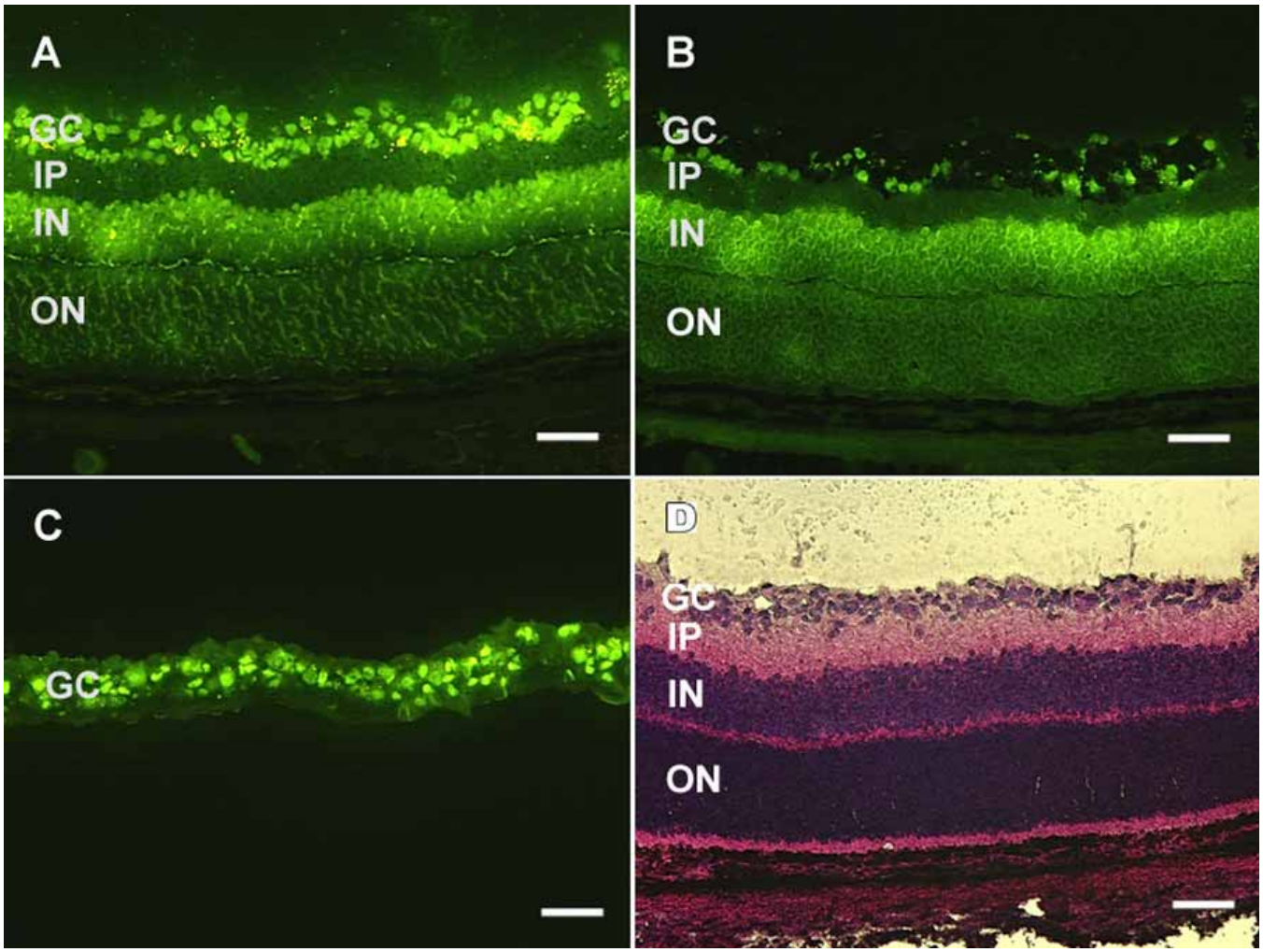


Figure 1. Visualization of retinal ganglion cells and LCM. Retinal ganglion cells were retrograde labeled by injection of the fluorescent dye aminostilbamidine into the superior colliculus and visualized using fluorescent microscopy. **A**: The retinal ganglion cells are in the upper most fluorescent layer, with round cell bodies exhibiting a strong fluorescence. Background fluorescence allows the inner and outer nuclear layers to be distinguished. **B**: The same section as shown in A, after LCM. Most of the ganglion cells have been removed while the underlying inner nuclear cell layer is unperturbed. **C**: The post-LCM ganglion cells adhering to the underside of the capturing cap. **D**: HE staining of a retinal cryosection from the same rat pup. The scale bar represents 50 μm. The ganglion cell layer (GC), inner plexiform layer (IP), inner nuclear layer (IN), and outer nuclear layer (ON) are indentified.

Ca^{2+}) followed by ionomycin in the base solution without Ca^{2+} and with the addition of 5 mM EGTA (pH 8.0). The 340/380 ratio was converted to Ca^{2+} concentration as previously described [27]. All experiments were performed at 25 °C. All materials were from the Sigma-Aldrich Corp. (St. Louis. MO) unless otherwise noted.

RESULTS

Expression of A_3 adenosine receptor in cells isolated by LCM: Retinal ganglion cells comprise less than 5% of the total retinal cell population. As ganglion cells are sandwiched among the inner retinal neurons, glial cells, and the inner limiting membrane, obtaining samples of sufficient purity for molecu-

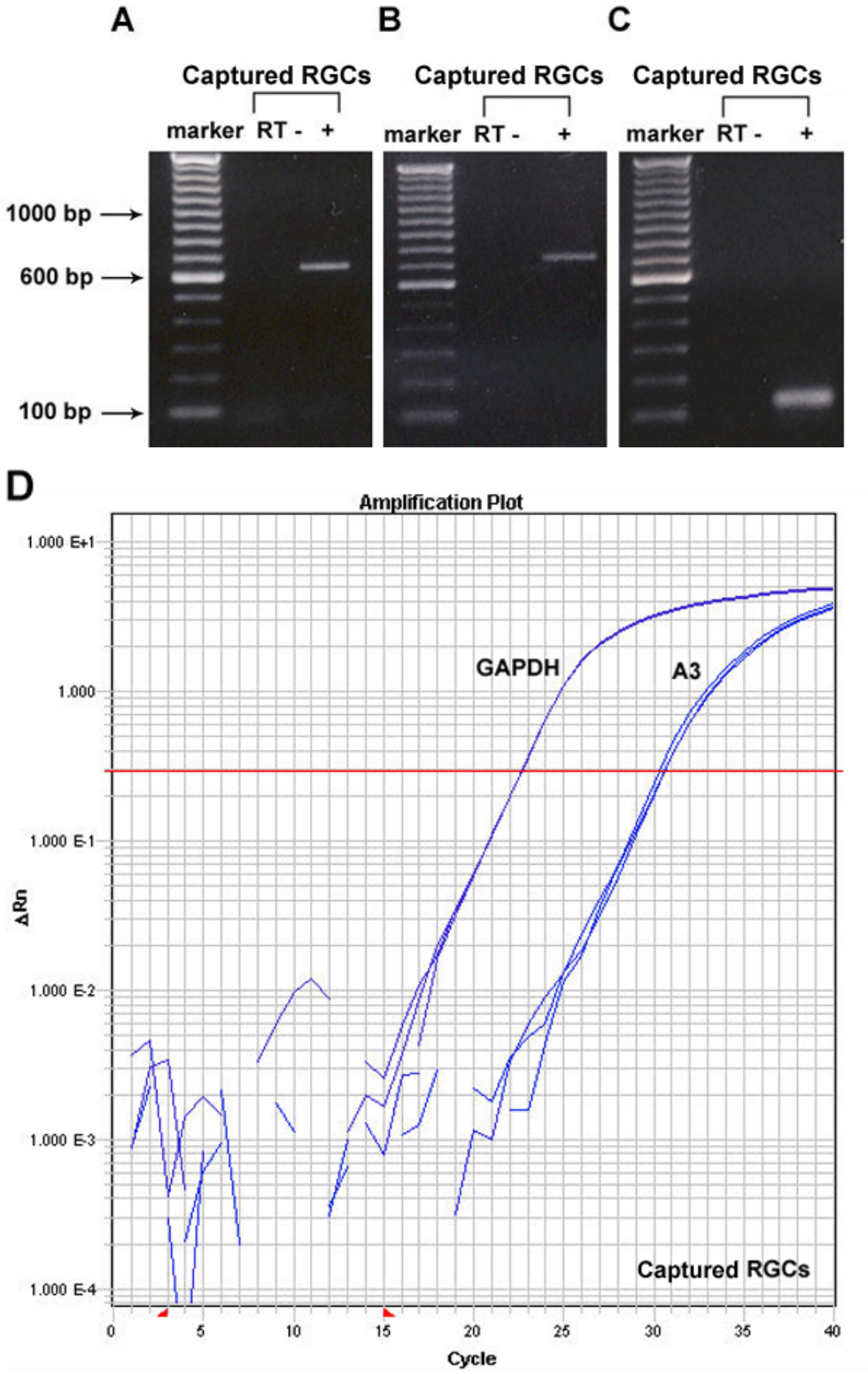
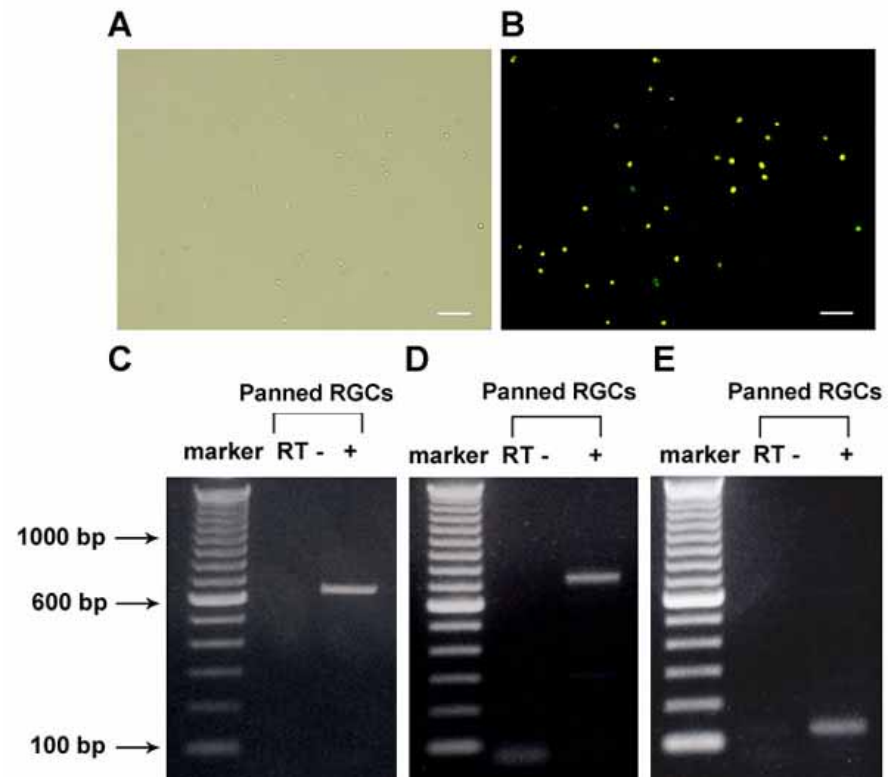


Figure 3. Gene expression of A_3 adenosine receptor in captured cells from the ganglion cell layer. Expression of A_3 adenosine receptor mRNA was detected from material obtained from labeled cells in the ganglion cell layer. Panels **A**-**C** shows results from traditional PCR run on 1% agarose gels. **A**: The 641 bp PCR product of the 5' end of the encoding sequence from primer pair A_3U is shown. **B**: The 724 bp PCR product of the 3' portion from primer pair A_3L. **C**: The 123 bp PCR product of the cDNA of A_3 adenosine receptor using the primers for real-time PCR. The left lanes correspond to PCR marker; size of the DNA fragments is indicated on the left. Negative controls, obtained by omitting the reverse transcriptase, are indicated by "RT-". **D**: A typical result from real-time PCR analysis using primers for the GAPDH and A_3 receptor genes. The abscissa indicates the number of PCR cycles and the ordinate shows the fluorescence emitted by SYBR green dye-labeled PCR products. The curves on the left represent the PCR product of GAPDH and the curves on the right represent the PCR products of A_3 adenosine receptor. Each reaction has been performed here in triplicate. Similar results were found with RNA from 5 different microdissections.



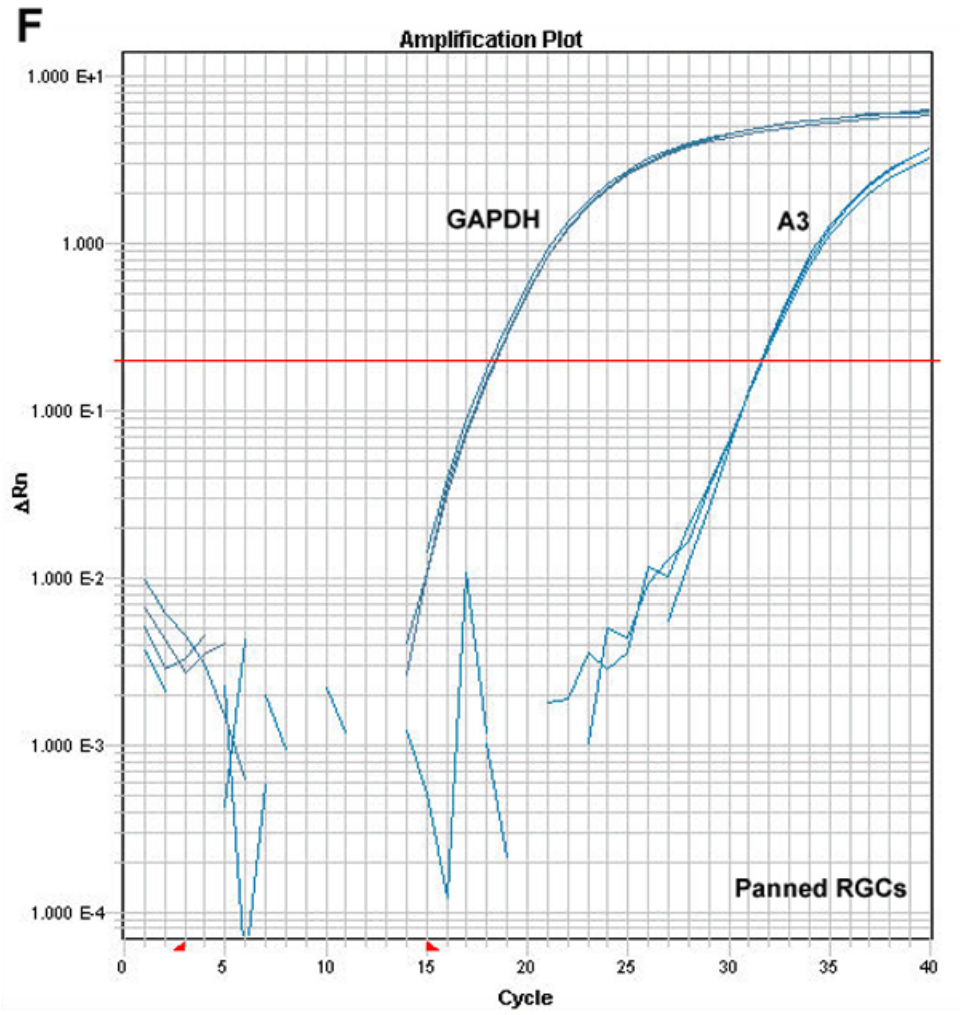


Figure 4. Gene expression of A_3 adenosine receptor in the retinal ganglion cells harvested by immunopanning. **A**,**B**: Retinal ganglion cells images taken 1 h after immunopanning. Panel **A** shows a brightfield image, while Panel **B** shows the same cells observed under fluorescent light. All cells present in the field are fluorescent, indicating the high purity of the harvest (bars represent 100 μm). **C**-**E**: The expression of the A_3 receptor mRNA in isolated ganglion cells detected with conventional RT-PCR. **C**: The 641 bp product of primer pair A_3U. **D**: The 724 bp PCR product from primer pair A_3L. **E**: The 123 bp PCR product of pair A_3Q. **F**: The expression of the A_3 receptor mRNA in purified ganglion cells using real-time RT-PCR, as described for Figure 3D. Similar results were found with cDNA obtained from 5 separate isolations.

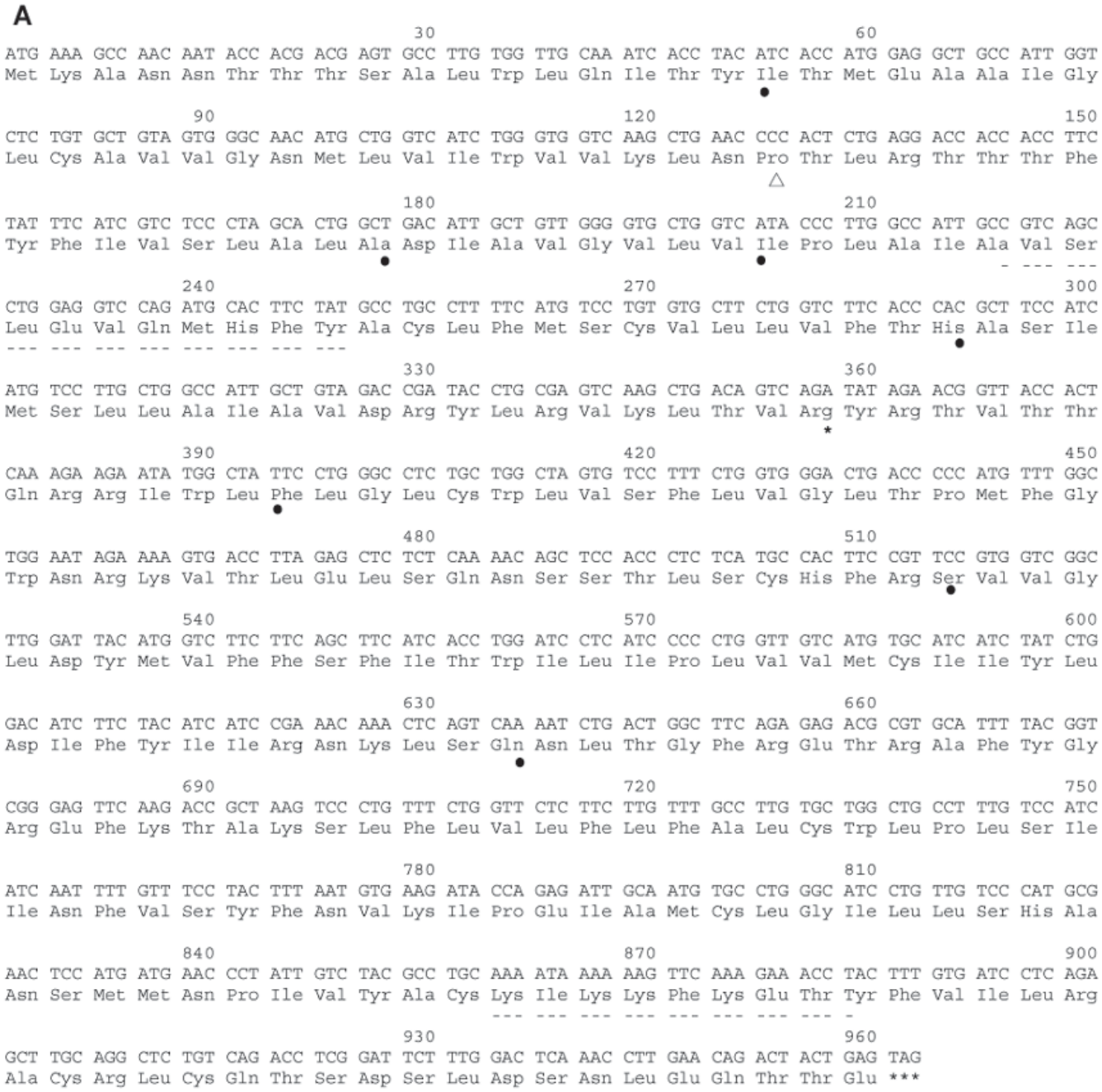


Figure 5. Nucleotide sequence and amino acid alignment of rat A_3 adenosine receptor coding region. **A**: The nucleotide sequence and corresponding amino acid alignment of the rat A_3 adenosine receptor coding region in our study, and compares the nucleotide alignment with other sequences. DQ075463 is the sequence of our material; X95249 is the first published A_3 sequence [33]; M94152 is the sequence which is first identified as A_3 receptor [32]; and X93219 is the sequence with a splice variant [34]. Differences with the other sequences are illustrated; conservative nucleotide differences between our sequence and all three other sequences are indicated by a black dot below the corresponding nucleotide. At position of 128, G in the was replaced by C, which resulted in the substitution of amino acid change from arginine to proline (indicated by "Δ"). The start of the splice variant is indicated by asterisk. Note that X59249 has a deletion of C at position of 218 and an insertion of G to put the sequence back in frame, and M94152 has an insertion of A at position of 858 and a deletion of T at position 883 (both indicated by a dashed underline). **B**: The amino acid sequences of all four variants are displayed along with the consensus sequence. Deviations from the consensus are highlighted in a box. The splice variant in X93219 begins after amino acid 119. Of the four sequences, DQ075463 most closely resembles the consensus sequence.

lar analysis is difficult using standard dissection techniques. In present study we used both LCM and immunopanning to concentrate genetic material from retinal ganglion cells in order to search for the A_3 adenosine receptor.

Several days after the dye aminostilbamidine was injected into the superior colliculus, the spherical cell bodies of retinal ganglion cells fluoresced yellow-green, with retinal whole mounts indicating dense and even staining. As illustrated in Figure 1A, this yellow staining of ganglion cell bodies was evident within the laminar organization of the retina. Only large, fluorescent cell bodies were "captured" or collected with the laser, and those in close proximity to unlabeled material were left behind to avoid obtaining material from unlabeled cells. However, the 7.5 μm width of the laser made it difficult to ensure that captured material was exclusively from ganglion cells. Figure 1B shows the same retinal section after removal of material from the ganglion cell layer, while Figure 1C shows the selected cells adherent to the underside of the capturing cap. Corresponding retinal anatomy from an adjacent section after HE staining is illustrated in Figure 1D.

Expression of A_3 adenosine receptor mRNA in retinal ganglion cells isolated by LCM was detected using traditional RT-PCR. Two sets of primers, A_3U (upper) and A_3L (lower), with a 173 bp overlapping sequence, were used to amplify the entire encoding region (Figure 2). Bands of the predicted product sizes were detected; 641 bp for A_3U and 724 bp for A_3L (Figure 3A,B). Analogous bands were observed in three replicates. No product was detected when RT was omitted from the reaction as a negative control.

Amplification of mRNA obtained from the total retina of rat pups and from the testes of adult rat also revealed bands of 641 bp and 724 bp using primer pairs A_3U and A_3L. When sequenced, the products from the total retinal and testes material maintained the distinct sequence found in retinal ganglion cells, with complete identity in samples from all three sources.

Confirmation of the A_3 message in captured cells from the ganglion cell layer was obtained using real-time PCR. Product was detected from 5 captured preparations using A_3Q

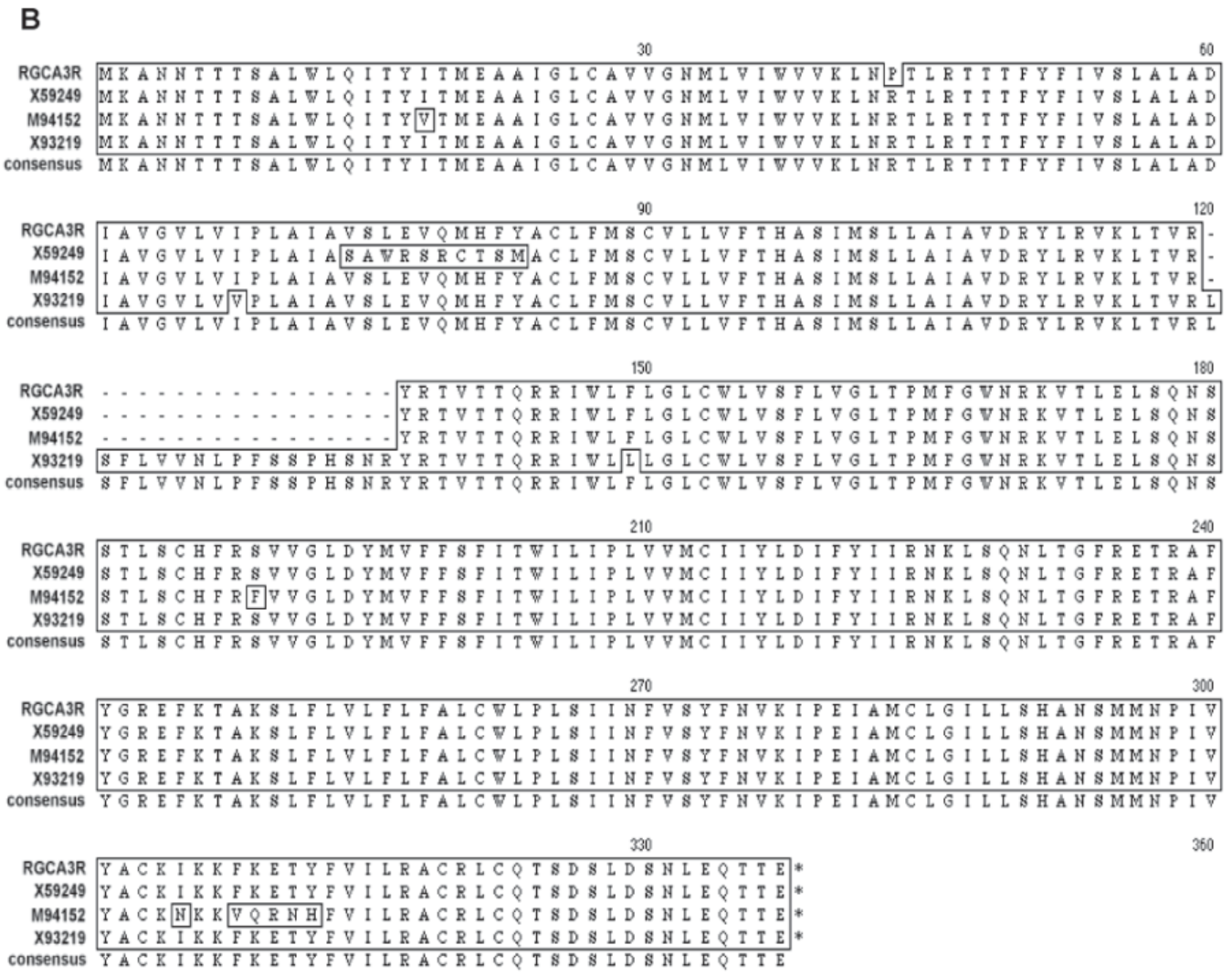


Figure 5. Contnued.

(quantitative) primers described in Table 1. The dissociation curve run after the reaction indicated the products of the GAPDH and A_3 primers were not contaminated, and that no product was detected in the absence of template. The specificity of these primers was confirmed by using them for traditional PCR (Figure 3C) and sequencing the product. Qualitative analysis suggested the A_3 message was present in relatively low abundance, with product crossing threshold 7.0±0.8 (n=5) cycles after that detected with GAPDH primers. While this analysis does not enable predictions of relative copy number, it is consistent with relatively small amounts of mRNA for the A_3 receptor.

Expression of A_3 adenosine receptor in immunopurified ganglion cells: While the large, fluorescently labeled cell bodies in the ganglion cell layer were most likely ganglion cells, the imprecision of the LCM laser, combined with the presence of amacrine, glial, and endothelial cells in the ganglion cell layer made it possible that material from these cells con-

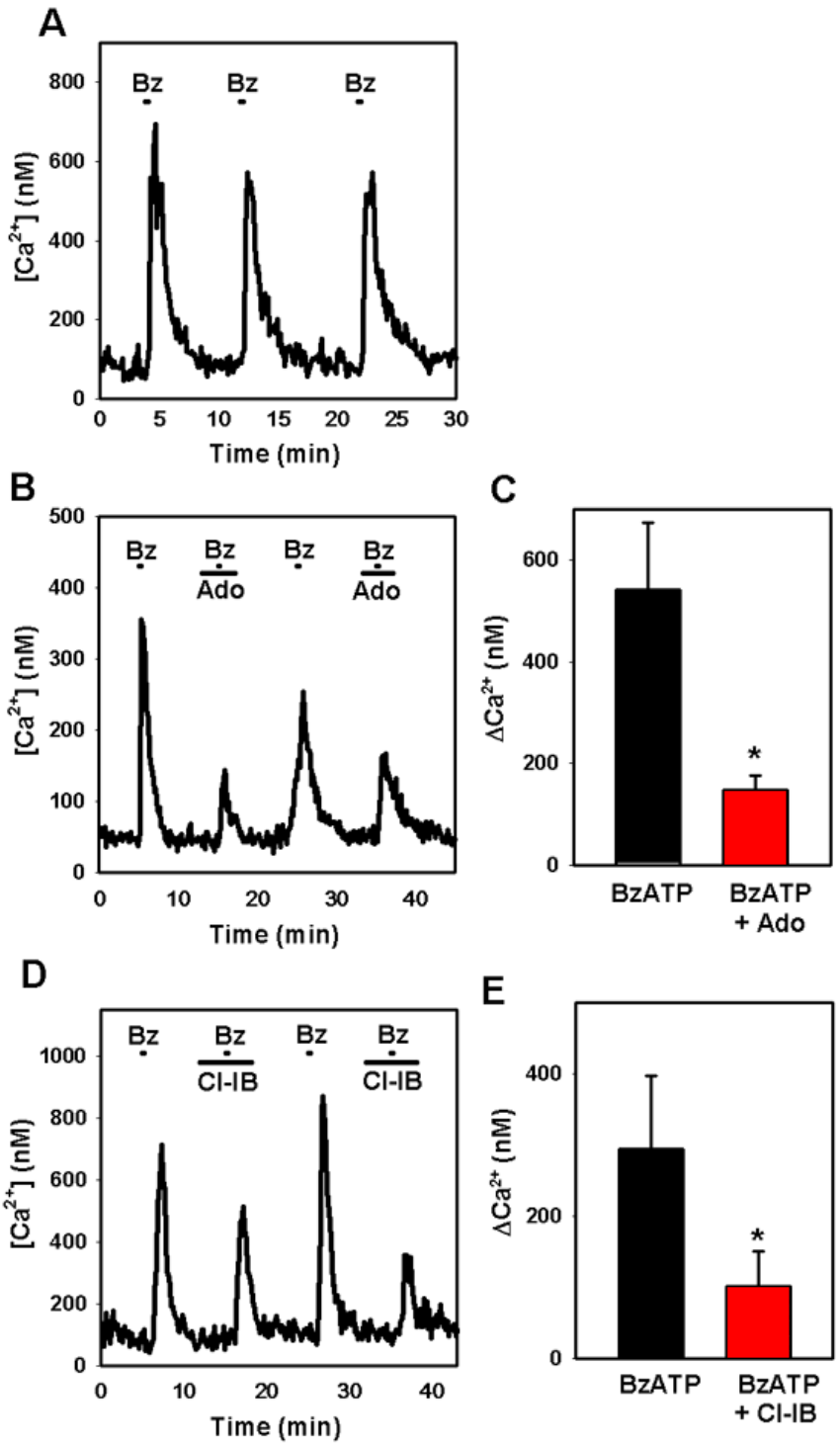


Figure 6. Effects of A_3 receptor activation on immunopurified ganglion cells. **A**: The $P2X_7$ receptor agonist BzATP (50 μM) led to large, repeated increases in Ca^{2+} when given in 15 s applications with an 8-10 min wash in between. **B**: Application of 10 μM adenosine attenuated the response to BzATP. Adenosine was given 2 min before and 2 min after the BzATP to maximize effectiveness. **C**: Adenosine reduced the mean Ca^{2+} response to BzATP. To control for slight desensitization, the first and third application were averaged together to obtain each value for "BzATP", while the second and fourth responses were averaged to obtain each "Bz+ Ado" level. The bars represent mean±SEM from 12 ganglion cells. The asterisk in this panel signifies p=0.003 (paired Student's t-test). **D**: The A_3 agonist Cl-IB-MECA (1 μM) produced a similar block of the BzATP response. **E**: Cl-IB-MECA also reduced the mean response to BzATP. Ca^{2+} elevations were averaged as in Panel **C**. The asterisk in this panel signifies p=0.019, n=6 (paired Student's t-test).

tributed to the mRNA used above. To confirm that mRNA for the A_3 receptor was present in ganglion cells, a two-step immunopanning technique was used to obtain a purified population of ganglion cells. When retinas containing labeled ganglion cells were used, >98% of the immunopanned cells were fluorescent, indicating a high degree of purity (Figure 4A,B). RNA extracted from immunopanned cells displayed bands of appropriate size using A_3U, A_3L, and A_3Q primers (Figure 4C-E). Sequencing found the product 100% identical to the message from microdissected cells. Product was amplified from cDNA obtained from each of 5 independent preparations of panned cells using real-time PCR (Figure 4F). The amplification product crossed threshold at 13.0±0.2 (n=5) cycles after that detected with GAPDH primers. Again, the dissociation curve verified the purity of the product found with GAPDH and A_3 primers, and that no product was found in the absence of template.

Comparison of A_3 sequence: The sequence of the encoding region from ganglion cell layer and purified ganglion cells was over 99% identical to sequences for the rat A_3 receptor with accession numbers M94152 [32] and X59249 [33], although several single nucleotide alterations and a few more extensive changes were present. The unique nucleotide sequence obtained in the present study is found in Figure 5A, and was entered into the Genbank database with accession number DQ075463. Most of the single substitutions were conservative, but G128C resulted in the substitution of a basic arginine to a nonpolar proline in the first intracellular loop. The sequence differed from X59249 at base pairs 219-249, and differed from M94152 at base pairs 859-883. The A_3 receptor in retinal ganglion cells shares both these regions with the splice variant of this gene expressed in brain cells (accession number X93219) [34], although the spliced insertion was not present in our material. The resulting comparison of these four amino acid sequences for the A_3 receptor is illustrated in Figure 5B.

Functional identification of the A_3 receptor in immunopurified cells: A_3 receptor agonists can protect ganglion cells present in a preparation of mixed retinal cells from stimulation of the $P2X_7$ receptor agonist [24]. However, it is not known whether these agonists can act directly on the ganglion cells. The protective effect of the specific A_3 receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyluronimide (Cl-IB-MECA) [8] was thus examined on immunopurified retinal ganglion cells with the Ca^{2+} sensitive dye fura-2. Repetitive 15 s applications of 50 μM 3'-O-(4-benzoylbenzoyl)ATP (BzATP) led to large, repeatable elevations in the Ca^{2+} levels of isolated ganglion cells (Figure 6A). While BzATP can activate several P2 receptors, previous characterization has demonstrated that its actions are mediated by the $P2X_7$ receptor in retinal ganglion cells [27]. The rise in Ca^{2+} triggered by BzATP was prevented by 10 μM adenosine (Figure 6B), with a mean decrease of 65.8±3.8% (n=12; Figure 6C). Of particular note, the A_3 receptor agonist Cl-IB-MECA (1 μM) also produced a substantial inhibition of the Ca^{2+} rise in isolated ganglion cells (Figure 6D). The block was reversible, repeatable, and significant, with a mean decrease of 69.3±4.8% (n=7; Figure 6E). Approximately 80% of purified ganglion cells responded to Cl-IB-MECA with some degree of block.

DISCUSSION

This study has demonstrated the presence mRNA for the A_3 adenosine receptor in genetic material isolated from the retinal ganglion cell layer using LCM microdissection and from immunopurified retinal ganglion cells. mRNA was detected using conventional PCR and confirmed with sequencing and real-time amplification. The message was distinct from previously reported rat A_3 sequences, with small differences throughout the reading frame. Receptor identification was supported pharmacologically with the A_3 receptor agonist Cl-IB-MECA. These data confirm the A_3 receptor in rat retinal ganglion cells on a molecular level and emphasize the protective opportunities of receptor activation.

In the past, detection of A_3 mRNA from retinal ganglion cells has been hampered by both the low copy number and the difficulty in obtaining a pure population of ganglion cells. The combination of LCM, immunopanning, and real-time PCR are well suited to address both concerns and provide direct information about gene expression in these cells. The application of LCM to labeled ganglion cells in rapidly frozen tissue sections provided genetic material from the ganglion cell layer. While the presence of additional cell types in this layer may have diluted the purity of the captured sample somewhat, the ability to select large, fluorescent cell bodies with the laser will have increased selectivity. The detection of mRNA for the A_3 receptor in immunopanned cells provides an additional level of specificity. However, the 4-6 h required to isolate ganglion cells with the immunopanning technique is not ideal, and may have been sufficient to enable mRNA degradation. Although prediction of copy number is not possible without standard curves constructed from known quantities of mRNA, the relative expression of the A_3 mRNA with respect to GAPDH suggests the captured material had more mRNA than panned cells. As the captured material was quick frozen, it is possible that the A_3 mRNA was particularly sensitive to degradation.

The relatively low expression of the mRNA for the A_3 receptor in both preparations is consistent with two previous studies that failed to detect A_3 mRNA in the entire eye [25] or in the retina itself using in situ hybridization [26]. The presence of the A_3 receptor was subsequently confirmed in the ciliary epithelium using functional and molecular tools [35,36], implying the in situ technique was not sufficiently sensitive to detect message present in low copy number in other ocular tissues. This observation in the eye agrees with that found elsewhere, with high levels of expression primarily confined to the testes [9,26,32]. Previous attempts to overcome this difficulty in the retina examined the activity of the A_3 receptor promoter linked to a β galactosidase reporter gene in transgenic animals. β galactosidase staining revealed promoter activity in the cerebrospinal vasculature and in a subset of ganglion cells [26]. Although staining was only observed in <10% of the total ganglion cells, this could underestimate of the pro-

portion of cells expressing the A_3 gene if the turnover rate of the mature mRNA is low or if transgene expression was variable. Pharmacologic analysis indicates that A_3 agonist Cl-IB-MECA was effective on most immunopanned ganglion cells, suggesting the receptor itself is widely expressed.

The A_3 receptor genes obtained from ganglion cells, whole retina and testis were identical to each other, but unique from other published sequences for the rat A_3 receptor. Of the four sequences examined, ours appeared to reflect the baseline sequence, with each of the three larger changes occurring in just one of the other three sequences. The insertion of an additional 51 base pairs in an alternatively spliced variant cloned from rat brain (A3i; X93219) was associated with reduced coupling to G_i proteins [34]. This insertion was not present in our samples, implying activation of the A_3 receptor will lead to more robust activation of G_i in our preparation. According to the predicted transmembrane topology of the A_3 receptor [34], the differences between our sequence and X59249 affect the entire first extracellular loop, while those between our sequence and M94152 affect the cytoplasmic region just after the seventh transmembrane domain [32,33]. Although functional contributions of these regions, or these corresponding changes, are not presently understood, the shift from X59249 to our sequence removed a putative PKC δ phosphorylation site and this may have functional consequences. On a broader scale, the sequence changes illustrate the presence of different sequences in different rat strains, and emphasize caution when comparing pharmacologic or physiologic data from different laboratories.

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