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GluA2 AMPA glutamate receptor subunit exhibits codon 607 Q/R RNA editing in the lens

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Abstract

Regulated GluA2 AMPA subunit expression, RNA editing, and membrane localization are fundamental determinants of neuronal Ca²⁺ influx, and underlie basic functions such as memory and the primary brain disorder epilepsy. Consistent with this, AMPARs, and specifically GluA2, are targets of common antiepileptic drugs (AEDs) and antidepressants. Recently, epidemiological associations between epilepsy and increased cataract prevalence were found comparable to cataract links with diabetes and smoking. Similarly, use of AEDs and several antidepressants also showed links with increased cataract. Here, we demonstrated GluA2 in lenses, consistent with REST/NRSF and REST4 we described previously in lenses, as well as GluA1 and ADAR2 in the lens. Surprisingly, we found predominant neuron-like Q/R editing of GluA2 RNAs also occurs in the lens and evidence of lens GluA2 phosphorylation and STEP phosphatases linked with GluA2 membrane localization in neurons. This study is among the first to show GluA2 expression and predominant Q/R RNA editing in a non-neural cell. Our results suggest GluA2 AMPARs have related roles in lens physiology and disease processes, and provide evidence these anticonvulsant and antidepressant drug targets also occur in the lens.

Epidemiological studies found that epilepsy and use of several antiepileptic drugs (AEDs) are associated with increased cataract prevalence (20,23,25,34,42,46). Similarly, several antidepressants were linked with increased cataract formation (16,36,49). Epilepsy and these drug targets were shown to involve glutamate receptors, and in particular the GluA2 (GluR2) subunit of glutamate AMPA receptors (AMPARs) (4,6,26,40,43,47,52,53). Epileptic seizures, which are considered the most common forms of primary brain disease, result from over-synchronous firing of neurons and can be initiated and propagated by AMPAR stimulation (44). The present study examined these relationships between lens and brain, and determined if GluA2 expression and its RNA editing also occur in the lens.

The current understanding of AMPARs is largely derived from their role as the predominant mediator of excitatory neurotransmission in the central nervous system (40). GluA1-4

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(GluR1-4) assemble in various combinations that confer substantially different Ca^{2+} permeability on AMPARs, with GluA2 having a primary role (55). In the brain, essentially all AMPARs contain GluA2 edited by ADAR2 (Adenine deaminase, RNA specific) at codon 607 within the Ca^{2+} pore (3,7,22,54). Conversion of this Adenine to Inosine (utilized as Guanine) causes Arg to be substituted for Gln and blocks Ca^{2+} entry. This limits potentially toxic shifts in Ca^{2+} in relatively quiescent neural cells, which can also occur in the lens (15,19,41,45). GluA2 under-editing will also act as a genetic switch for seizures (22,26). For example, a gene mutation at codon 607 which eliminates its editing and remains a Gln codon, causes epilepsy and is lethal by three weeks of age in mice (10). Ca^{2+} entry in neurons regulated at the level of GluA2 expression and RNA editing also has a fundamental role in long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission that underlie memory formation as well as mood (4,17,21,38). GluA2 under-editing is also key in amyotrophic lateral sclerosis and associated cell loss (3,54,60). Since glutamate is the principal excitatory neurotransmitter, glutamate receptors that include AMPARs are important in initiating and spreading of seizures, and AMPAR antagonists are commonly used as AEDs. In addition, endocytosis determines AMPAR surface populations to regulate LTP and LTD (2,4,5), and GluA2 phosphorylation and its RNA editing also regulate these processes in neurons (3,17,21,51). GluA2 membrane localization is enhanced by C-terminal tyrosine phosphorylation, and STEP proteins (striatal-enriched phosphatases) were identified that remove these phosphates (58). The antidepressant fluoxetine affected AMPARs by increasing GluA2 and altered AMPAR subunit ratios in brain, which is thought to contribute to its psychotropic properties (4,57). In culture, fluoxetine and other antidepressants augmented GluA2 RNA editing (47). Thus, edited GluA2 is linked with normal low Ca^{2+} permeability, and suppression of GluA2 and its RNA editing can contribute to disease. We note that calcium channels, connexins, serotonin receptors, as well as sodium and potassium gating proteins, also regulate ion homeostasis in lens and brain (13,14,19,41), and implicated in AED and antidepressant actions. However, in addition to cell-cell communication, glutamate and AMPARs regulate neural development and plasticity, and dendritic spine shape and density (35), which may also have significance in the lens in light of detailed similarities described between neuronal dendritic spines and lateral protrusions on lens fiber cells (59), and also consistent with roles for miR-124, let-7 and miR-125 in dendrite regulation which we also identified in the lens (8,9,18).

Further parallels between lens and brain are seen in the suppression of GluA2 expression by REST/NRSF (RE-1 Silencing transcription Factor/Neuron Restrictive Silencing Factor) (11,12,32,37). Previously, we showed REST/NRSF also decreases in lens fiber cells, similar to its role in neuronal differentiation (8,9,33). Consistent with these observations, increased and/or ectopic REST/NRSF due to ischemia and stress was linked with corresponding GluA2 decreases in epilepsy and other forms of brain injury and disease, and identified increased Ca^{2+} entry as a key factor in neurotoxicity (7,12,29,39,50). By contrast, ADAR2 is expressed in many tissues with a large number of editing substrates. Although ADAR2 deficiency produces lethal seizures in mice, mutation of GluA2 codon 607 from Gln to Arg at the DNA level rescued ADAR2^{-/-} mice, and identified GluA2 as the physiologically most important ADAR2 substrate (26).

Materials and Methods

Animals and tissue use

Mouse and rat tissues were used according NIH guidelines. GluA2^{-/-} mouse tissues were the gift of E. Kavalali (UT Southwestern, TX). Lens and brain samples were extracted in Trizol (Invitrogen) for RNA preparation, or placed in SDS buffer with protease inhibitors (Roche) and phosphatase inhibitors (Sigma) for protein analysis. Enucleated eyes were

placed in buffered 4% paraformaldehyde for 24–48 hours to prepare paraffin sections for histology.

Immunoblotting and immunofluorescence

Total proteins resolved on Tris-bis gels (Invitrogen) were blotted to filters. Filters blocked in 5% NFDM/PBS were probed with antibodies diluted according to the manufacturer. After washing, filters were re-probed with HRP-2^o antibodies (Jackson Immunologicals) and visualized by chemiluminescence (Amersham). For immunofluorescence studies, eye sections de-waxed in xylenes and alcohol washes were blocked in 10% goat serum/PBS. Sections probed overnight at 4°C were subsequently probed with fluor-conjugated 2^o antibodies (Invitrogen). Antibodies included anti-GluA1 (Genscript), anti-ADAR2 (Sigma), anti-GluA2 mAb (Millipore), anti-GluA2 (Epitomics), anti-Phospho GluA2 Y869/873/876 (Cell Signaling), anti-GluA2 (Millipore), 23e5 mAb anti-STEP (Novus Biologicals), and anti-STEP (Cell Signaling).

Analysis of RNA editing

Total RNA from 4 week-old mouse and rat lenses was used to produce cDNA. Nested primers described elsewhere (24), were used to amplify GluA2 transcripts from the edited transcript region with the following sequences: F5'-TCTGGTTTTTCCTTGGGTGCC-3', *F5'-GGTTTTTCCTTGGGTGCCTTTAT-3', R5'-AGATCCTCAGCACTTTCG-3', *R5'-ATCCTCAGCACTTTCGATGG-3'. Primers spanned exons 11 and 12 to ensure products were derived from mRNAs. Products resolved on agarose gels were purified with kits (Qiagen) and re-amplified with nested primers, and their nucleotide sequences were determined to produce color-coded histograms. In addition, the restriction enzyme Bbv1 (NE Biolabs) was used to test for loss of a recognition site due to RNA editing (39).

Results

To test for AMPAR subunit expression in lenses we used GluA1 and GluA2 specific antibodies to probe protein samples from 4 week-old mouse lenses on immunoblots, and to identify their protein distributions in histological sections (Fig. 1). *In situ* studies showed signals for GluA1 and GluA2 that indicated they were predominantly expressed in nucleated elongated fiber cells at the lens periphery (lens cortex). Both subunit proteins decreased in expression in the lens interior, at a point where fiber cell terminal differentiation initiates and fiber cell organelles are lost to form the lens nucleus. In addition, GluA2 signal was present in adjacent retinal layers in the same sections, comparable to studies by others in the retina (48). Likewise, immunoblots detected both AMPAR subunits in the lens and brain. We next examined lenses from *wt* and GluA2^{-/-} mice. Using anti-GluA2 antibodies *in situ* we detected GluA2 in *wild-type* but not GluA2 deficient mouse lenses (Fig. 1, panel III). In addition, ADAR2 was also identified in lenses that would be required for GluA2 RNA editing (Fig. 1). The distribution of ADAR2 in mouse lens cells was consistent with descriptions by Kwak and coworkers in their examination of ADAR2 in spinal cord neuronal cells (3).

We next examined lenses to determine if C-terminal tyrosine phosphorylation of GluA2 occurs in lenses similar to the brain. We detected this Tyr-phosphorylated GluA2 epitope largely in cortical fibers in *wt* lenses (Fig.2), but not in GluA2^{-/-} lenses similar to detection of GluA2 in knockout lenses above (not shown). We also examined lenses for expression of striatal-enriched tyrosine phosphatase (STEP). STEP was shown to remove these phosphate modifications from GluA2 in neurons (58). When we probed immunoblots with mouse and rabbit anti-STEP we detected STEP46 and STEP61 isoforms in lens and brain protein samples (Fig. 2, panel II), and *in situ* we detected STEP more strongly in cortical lens fiber

cells, which overlapped with detection of GluA2 and Phospho-Tyr GluA2 epitope in mouse lenses (Fig. 2).

These findings encouraged us to examine lenses to determine if GluA2 RNA editing also occurs in this non-neural tissue. Total RNA from rat and mouse lenses was used to amplify GluA2 transcripts from the region encoding the Q/R codon 607 edited site. Nested GluA2 primers were used that corresponded to adjacent exons encompassing the codon 607 region, and ensured amplification of cDNA (Fig. 3). When the nucleotide sequences of these products were determined, the resultant histogram showed Guanine was the predominant nucleotide identified at this position in GluA2 transcripts from mouse and rat lenses (Fig. 3). This result indicated that RNA editing at codon 607 also occurs in the lens, and consistent with the interpretation that GluA2 transcripts are predominantly edited at this site in the lens as well. Using a second method to determine the nucleotide at the edited site, we found that digestion of amplified products from this transcript region with Bbv1 (39) identified only one cleavage site, and agreed with analysis of GluA2 editing in neural cells (54).

Discussion

Our study identified GluA1 and GluA2 expression in the lens, and surprisingly found that GluA2 is predominantly edited at a critical Ca^{2+} pore position in the lens, analogous to neural cells. This editing event is consistent with ADAR2 we also observed in lenses. However, additional studies will be required to determine if functional AMPARs form on lens cell surfaces containing edited GluA2. We also found evidence that GluA2 C-terminal tyrosine phosphorylation occurs in lenses, which also appeared consistent with identification of STEP in the lens. These findings suggest GluA2 phosphorylation and STEP proteins may also function similarly in lens to determine GluA2 membrane localization. STEP46 was characterized as cytosolic, whereas STEP61 is targeted to post-synaptic sites in neurons, and future studies can determine if similar distributions occur in lens cells (58). Together, GluA2 expression and predominant Q/R RNA editing, Tyr-phosphorylation and STEP expression in lenses lead us to speculate that GluA proteins and specifically GluA2 have related roles in lens physiology and disease.

Regarding the tissue distribution of GluA2 Q/R RNA editing, an examination of the literature indicated this editing event has been studied extensively over the past 20 years and has only been described in neural cells to date. GluA2 Q/R editing also occurs in pancreatic ganglion cells which also have neural origins (31). We note that GluA2 detected largely in lens fiber cells agrees with decreased REST/NRSF we showed previously in fiber cells (8,9), and indicates further investigations are warranted to determine if a similar relationship between REST/NRSF regulation and GluA2 occurs in this additional organ. Several studies linked ectopic REST expression with stress in the nervous system, which led to a suppression of GluA2 and a corresponding increase in Ca^{2+} permeability, and suggests stress may also contribute to related loss of calcium homeostasis in cataracts (1,56).

The present findings also appear consistent with Gamma-aminobutyric acid (GABA), GABA receptors and GABA-related enzymes expressed in the lens (27,28,30). GABA is the major inhibitory neurotransmitter and also linked with neural development and plasticity. These observations suggest Glutamate and GABA have corresponding antagonistic roles in the lens linked with the precise cell growth needed to form an optically useful organ in proper alignment with the cornea and retina, and coordinated with expression of additional channel proteins and connexins needed to regulate ion homeostasis. In summary, our results suggest that GluA2 dysregulation may contribute similarly to a loss of Ca^{2+} homeostasis in this additional organ. These findings also indicate that shared expression and regulation of GluA2 in lens and brain may be a common factor contributing to increased cataract

prevalence observed in epilepsy. Last, our study also identifies GluA2 expression and RNA editing in the lens as a potential off- target drug interaction site also involved in increased cataract occurrence associated with AED and antidepressant drug use.

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Highlights

We examined AMPA glutamate receptor subunit expression in the lens.

We showed GluA1, GluA2, as well as ADAR2 and STEP phosphatases in the lens.

Further, we demonstrated GluA2 Q/R RNA editing in this non-neural tissue.

This study appears first to identify GluA2 RNA editing outside the neural system.

We showed this anti-epilepsy and antidepressant drug target also occurs in the lens.

Fig. 1. Glutamate AMPA receptor subunits GluA1/GluA2, and RNA specific Adenine deaminase ADAR2 are expressed in *wt* mouse lenses

I: Immunofluorescence detection of protein in the lens, A: anti-GluA2, B: anti-ADAR2, and C: anti-GluA1 in lens. D: no 1^o control. Each protein was observed strongly in nucleated fiber cells at the lens periphery (~100-150 μ m in width), and decreased in terminally differentiated fiber cells in the lens interior. **II:** Immunoblots of total protein samples identifying protein expression in lens and brain, A: mAb GluA2, B: anti-GluA1, C: ADAR2, D: anti-GluA2; Le: lens, Br: brain samples. **III:** GluA2 was not detected in the lens in eye sections from GluA2^{-/-} mice, A: GluA2 detected in *wt* mouse lens, B: GluA^{-/-} lens, using GluA2 antibodies shown in panel II.

Fig. 2. Evidence C-terminal Tyrosine-phosphorylated GluA2, and STEP phosphatase linked with removal of Tyrosine phosphate modifications, occur in the lens

I: Detection of phospho-Tyr GluA2 epitope overlaps with GluA2 in nucleated cortical fiber cells. A,C. mAb anti-GluA2. B,D: anti-phospho-GluA2 (Tyr869/873/876). E: overlay of C,D: anti-GluR2 and anti-phospho-GluR2. **II:** Immunoblots identify Phospho-GluA2 (Tyr869/873/876) and STEP phosphatase proteins in the lens and brain, Left panel: Anti-Phospho-Tyr GluA2 detection. Right panels: Two antibodies detected proteins corresponding to STEP 61 and 46 kDa isoforms in the lens and brain. Color panel at far right shows amido black stained proteins. **III:** Anti-STEP antibodies detected STEP in cortical fiber cells at the lens periphery, overlapping with the distribution of GluA2 in lenses. A: 23e5 mAb anti-STEP, B: anti-GluA2, C: mAb anti-STEP. D: no 1° antibody.

Fig. 3. GluA2 transcripts expressed in mouse lenses predominantly undergo Q/R RNA codon 607 editing, shown to restrict calcium influx in neurons

I: Diagram of PCR strategy and agarose gel showing RT-PCR amplification of GluA2 cDNA from the region surrounding codon 607. PCR primers correspond to sequences in adjacent exons to ensure cDNA amplification. **II:** Histogram showing the nucleotide sequences of PCR products from the transcript region containing codon 607 from rat and mouse lens. The adenine present at this position at the DNA level is predominantly identified as guanine in amplified transcripts from mouse and rat lenses. **III:** Bbv1 digest of amplified products produced one cleavage site, consistent with RNA editing at the 5' restriction site.