

KCC2 expression supersedes NKCC1 in mature fiber cells in mouse and rabbit lenses

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Purpose: Na-K-Cl cotransporter 1 (NKCC1) and K-Cl cotransporter 2 (KCC2) have fundamental roles in neuron differentiation that are integrated with gamma-aminobutyric acid (GABA) and glutamate receptors, GABA synthesized by GAD25/65/67 encoded by *GAD1/GAD2* genes, and GABA transporters (GATs). Cells in the eye lens express at least 13 GABA receptor subunits, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) glutamate receptors, GAD1/GAD2, GAT1-4 and vGAT, and NKCC1. NKCC1:KCC2 ratios determine the switch in GABA actions from trophic/growth promoting early in development to their classic inhibitory roles in adult neurons. Lens epithelial cells cover the anterior surface and differentiate to elongated fiber cells in the lens interior with comparable morphology and sub-cellular structures as neurons. NKCC1 is expressed before KCC2 in neuron development and increases cell chloride, which stimulates differentiation and process formation. Subsequently, KCC2 increases and extrudes cell chloride linked with maturation. KCC2 has an additional structural moonlighting role interacting with F-actin scaffolding in dendritic spine morphogenesis. We examined KCC2 versus NKCC1 spatial expression in relation to fiber cell developmental status within the lens.

Methods: Immunofluorescence and immunoblots were used to detect expression in mouse and rabbit lenses.

Results: NKCC1 was restricted to peripheral elongating lens fiber cells in young adult mouse and rabbit lenses. Lens KCC2 expression included the major KCC2b neuronal isoform and was detected in interior fiber cells with decreased NKCC1 expression and localized at the membranes. Lens expression of RE-1 silencing transcription factor (REST) regulated KCC2 is consistent with GAD1 and GAD2, several GABA and glutamate receptor subunits, miR-124, and other REST-regulated genes expressed in lenses.

Conclusions: NKCC1 in peripheral elongating fiber cells is superseded by KCC2 expression in interior mature fiber cells that also express >20 additional integral GABA biology genes, AMPA/NMDA glutamate receptors, and an array of accessory proteins that together underlie morphogenesis in neurons. The present findings provide further evidence that this fundamental neuronal regulation is extensively conserved in lens and identify additional parallels in the morphogenetic programs that underlie lens fiber cell and neuronal differentiation and contribute to the development of visual acuity.

NKCC1 and KCC2 expression and functions underlie neuron differentiation and are increasingly a focus in studies of neurodevelopmental disorders [1-4]. NKCC1 increases cell chloride, which stimulates neuron differentiation and process formation [3,5]. Chloride has antiproliferative effects in neuronal precursors and alters growth factor actions from growth-promoting to stimulating neurite extension [6,7]. For example, KCl blocks neural progenitor DNA synthesis [6]. KCC2 extrudes cell chloride and is linked with neuron maturation [3]. NKCC1:KCC2 functions are integrated with gamma-aminobutyric acid (GABA) and GABA receptor biology and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate (NMDA) glutamate receptor developmentally regulated expression and functions. These factors determine the critical shift in GABA/GABA receptor polarity from trophic/growth promoting early in

development to their characteristic inhibitory functions in adult neurons [1,2,8-10]. GABA is synthesized in neurons by glutamate decarboxylase 1 (*GAD1*; OMIM: 60563) that encodes alternatively spliced GAD25 and GAD67 glutamate decarboxylases and glutamate decarboxylase 2 (*GAD2*; OMIM: 138275) that encodes GAD65 [11,12]. In addition, GABA transporters 1-4 (*GAT1-4* [OMIM: 137165, 603080, 615097, 607952] and vesicular *GAT* [OMIM: 616440]) are integrated into this process [11,12]. These interdependent genes continue to be under intense scrutiny to understand neural development as well as neurodevelopmental and neuropsychiatric disorders that include fragile X syndrome and related autism spectrum disorders and schizophrenia [3,4,13-15]. We examined the spatial expression of NKCC1 and KCC2 in relation to additional genes related to GABA biology in the lens.

Similarities between lens fiber cells and neurons include their elongated morphology and the ultrastructure of their vesicle transport machinery and lateral membrane protrusions/dendritic spines [16,17]. These observations are

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matched by extensively shared gene expression and use of fundamental interdependent molecular mechanisms shown to integrate their regulation at the DNA, RNA, and protein levels in neurons and initially described as distinguishing the neuronal phenotype [18-27]. This relationship is seen in the lens expression of the GABA-related genes cited above [11,12,25]. Lenses express NKCC1 [25], at least 13 GABA receptor subunits, GAD1, GAD2, GAT1-4, and vesicular GAT1 [11,12], as well as the major neuronal glutamate receptor subunits: NMDA receptor NR1, NR2A, NR2B, and AMPA receptor GluA1 and GluA2 [19,26]. The lens also expresses an extensive array of accessory signaling proteins consistent with GABA demonstrated in the lens [16-24].

Fiber cells in the lens interior form an axial microtubule membrane vesicle transport system as they elongate and produce arrays of membrane protrusions on their lateral surfaces [16,17,20]. Lens and neuron cell membrane spine protrusions have similar size, shape, and spacing and express many of the same scaffold, channel, receptor, and endocytosis-related proteins first described for their roles working with GABA and glutamate receptors at the neuronal membranes and dendritic spines [11,12,17,19,20-23, 25].

Neuronal NKCC1 protein is detected at birth in the rodent brain and increases to postnatal day (P)10-14 as the eyes open [3,27]. At that time, NKCC1 decreases relative to KCC2. KCC2 expression peaks at approximately day P14 and continues in the adult brain, linking KCC2 with neuron maturation. In humans the NKCC1:KCC2 ratios are high during embryonic brain development consistent with rapid growth and decrease to near parity after birth [3,4]. In neural system disorders, developmental delay in the transition from NKCC1 to KCC2 expression is linked with fragile X syndrome [3] and schizophrenia [4]. KCC2 also has a separate structural moonlighting role at neuronal membranes and specifically at the dendritic spines [28-30]. KCC2 interacts with F-actin scaffolding to constrain the protrusion structure and restricts lateral movement of surface proteins that include AMPA/NMDA glutamate receptors [28-30]. Thus, KCC2 is described as having two independent biological actions: as an ion transporter and as a structural protein orchestrating cytoskeleton organization at dendritic spines [3,5,6,27-30].

The lens is avascular, not innervated, and comprised of one cell type at successive developmental stages. Lens epithelial cells at the anterior surface exit the cell cycle at the anterior/posterior midline and begin a process of pronounced cell elongation as they move into the lens interior [16,17,20]. Fiber cell membrane spines express clathrin and AP-2 adaptor protein at their surface [17] that mediate AMPA/NMDA and GABA receptor internalization in neurons [20,24,26,31].

Lens fiber cells share expression of an additional group of membrane/dendritic spine proteins that include post-synaptic density protein 95 (PSD-95, DLG4), ephrin receptors, tyrosine phosphatase-STEP, p35, and Cdk5 cyclin dependent kinase, Ca(V) channels, neuronal c-src, and calcium calmodulin kinase II (CaMKII α) among others [16-26]. The expression of these proteins in the lens highlights the extent of parallels shown between fiber cell and neuron biology associated with the known roles of NKCC1, KCC2, and GABA and glutamate receptors in neuron development [1-4].

KCC2 gene expression is suppressed by REST transcription factors in neural progenitor cells and most non-neuronal cells, consistent with its initial characterization as neuron-specific [10,32]. A precedent for REST-regulated gene expression in lens is seen in the lens expression of *GAD1*, *GAD2*, *GluA2* (OMIM: 138247), *NR1* (OMIM: 138249), *NR2A* (OMIM 138253), *NR2B* (OMIM 138252), specific GABA receptor subunits, and includes miR-124 (OMIM 609237), L1 cell adhesion molecule (*LI CAM*; OMIM: 308840), and several other genes first studied for their roles in synaptic vesicle transport and dendritic spine morphogenesis [18-20,23-26]. The present study examined NKCC1 and KCC2 expression in young adult mouse and rabbit lenses.

METHODS

Tissues for study were obtained from New Zealand white rabbits about 4 months of age (Goffle Road Poultry Farms, Wyckoff, NJ) and 1-month-old C57Bl/6 mice (Charles River Laboratories, Wilmington, DE). These studies followed procedures approved by the Rutgers University institutional animal care and use committee and the ARVO Statement for Use of Animals in Research. Eyes and lenses for histological studies were fixed in 4% paraformaldehyde in PBS (0.01 M; 0.138 M NaCl; 0.0027M KCl at pH 7.4; Sigma Chemical Co., St. Louis MO) for 48 h before they were embedded in paraffin. Three lenses from the mice and rabbits were examined. Sections dewaxed in xylenes and graded alcohol washes were blocked in tris buffered saline (TBS; Sigma Chemical Co.) with 10% serum. Antibodies were applied at concentrations suggested by the suppliers in TBS/serum overnight at 4 °C. Fluor-conjugated secondary antibodies (Santa Cruz Biotechnologies, Paso Robles, TX, or Jackson Immunologicals, West Grove, PA) were then applied as suggested by the suppliers in TBS/serum. Negatived controls omitted primary antibodies and showed no signal, similar to Figure 1C. Antibodies were obtained from the following sources: KCC2, NKCC1, and GABBR1 mAbs from Neuromab/Antibodies Inc. (Davis, CA), anti-NKCC1 from Alamone Labs (Jerusalem, Israel),

and anti-KCC2b, anti-GABA β 3, and STEP mAb from Santa Cruz Biotechnologies.

Immunoblots used serially diluted total lens protein samples in sodium dodecyl sulfate (SDS) buffer resolved on filters. Filters blocked in TBS/non-fat dry milk were probed with antibodies as suggested by the suppliers. Immune complexes were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunologicals) and chemiluminescent detection (Millipore, Billerica MA) on the films.

RESULTS

NKCC1 and KCC2 have a fundamental role in neuron differentiation and subsequent maturation by determining cell chloride levels. They work with an array of GABA and glutamate receptors and associated membrane and dendritic spine proteins also shown to be expressed in the lens. We began by examining the expression of NKCC1 and KCC2 in young adult mouse lens. NKCC1 was previously identified in rodent lenses [25]. We detected the major neuronal isoform KCC2b in total protein samples of 1-month-old mouse lenses resolved by molecular weight (MW) on immunoblots (Figure 1).

We next determined the spatial expression and distribution of NKCC1 and KCC2 in mouse lenses. Immunofluorescence detection identified NKCC1 predominantly in peripheral actively elongating fiber cells closer to the lens perimeter using mAbs consistent with previous findings [25]. NKCC1 mAb and polyclonal antibodies identified this protein in differentiating fiber cells at the lens periphery. Extracellular domain-specific anti-NKCC1 antibodies identified NKCC1 at fiber cell membranes in the young adult mouse lens (Figure 1). KCC2 mAb and polyclonal anti-KCC2b antibodies also detected protein in peripheral elongating fiber cells in the mouse lens. However, fiber cells further in the lens interior showed KCC2 continues to be expressed in cells with little NKCC1 expression. In higher magnification views, KCC2 was detected primarily at the fiber cell borders of interior fiber cells, indicating a predominant membrane association in terminally differentiated mature fiber cells.

When we compared NKCC1 and KCC2 distribution in rabbit lenses, we observed similar expression patterns (Figure 2). NKCC1 was again detected predominantly in peripheral actively elongating fiber cells. KCC2 mAb and KCC2b antibodies detected protein in peripheral elongating fiber cells. Similar to findings in the mouse, NKCC1 expression was decreased in interior mature fiber cells in rabbit lenses, whereas KCC2 expression extended to fiber cells further in the lens interior and beyond the border where the NKCC1

levels showed a decrease. KCC2 was also observed to be relegated to the membrane borders between fiber cells in the mature rabbit lens fiber cells (Figure 2). Examination of fiber cells at higher magnification identified focal sites with higher concentrations of KCC2 along the perimeter of maturing fiber cells, and future ultrastructure studies can determine whether KCC2 concentrations are associated with fiber cell membrane protrusions.

Previous work by our group and others identified an array of additional neuronal proteins first shown to work with GABA and glutamate receptors at neuronal membranes and spines and expressed in the lens [16–24 and refs. within]. Our experiments showed that KCC2 colocalized in the mature fiber cells of rabbit lenses with striatal enriched tyrosine phosphatase (STEP; Figure 3) and the GABA β 3 subunit (Figure 3). In addition, KCC2 distributions in mature fiber cells also corresponded with detection of CAMKII α (Figure 4) at the level of light microscopy demonstrated in our laboratory.

DISCUSSION

An array of studies over the past 20 years have described detailed similarities between lens fiber cell and neuron morphology and sub-cellular structures that are matched by extensive parallels in their cell and molecular biology. The lens expresses at least 20 core genes in GABA biology that have an overarching role in neuron development as well as function. The present study extends these observations to NKCC1 and KCC2 in the lens. Our study showed mammalian lenses express KCC2 that includes the major neuronal KCC2b isoform in addition to NKCC1. We determined that NKCC1 is expressed as lens epithelial cells begin to elongate and move into the lens and subsequently decreases in mature fiber cells in the mouse and rabbit, again consistent with previous studies [25]. In contrast, KCC2 expression continues in mature fiber cells in the lens interior, beyond the border where fiber cells cease to elongate and enter terminal differentiation. Earlier studies that examined progenitor lens epithelial cells and cultures differed in results for KCC2 but did not examine fiber cells or intact lenses [33,34].

The present findings agree with the hypothesis that NKCC1:KCC2 expression ratios have a related role in lens fiber cell and neuron differentiation. Distributions of these antagonistic cotransporters in the lens are consistent with a role for increased cell chloride as fiber cell differentiation is initiated near the lens periphery, and later as KCC2 expression becomes predominant, with decreasing cell chloride levels as fiber cells mature in the interior of the lens. We speculate that cell chloride increased by NKCC1 at the lens

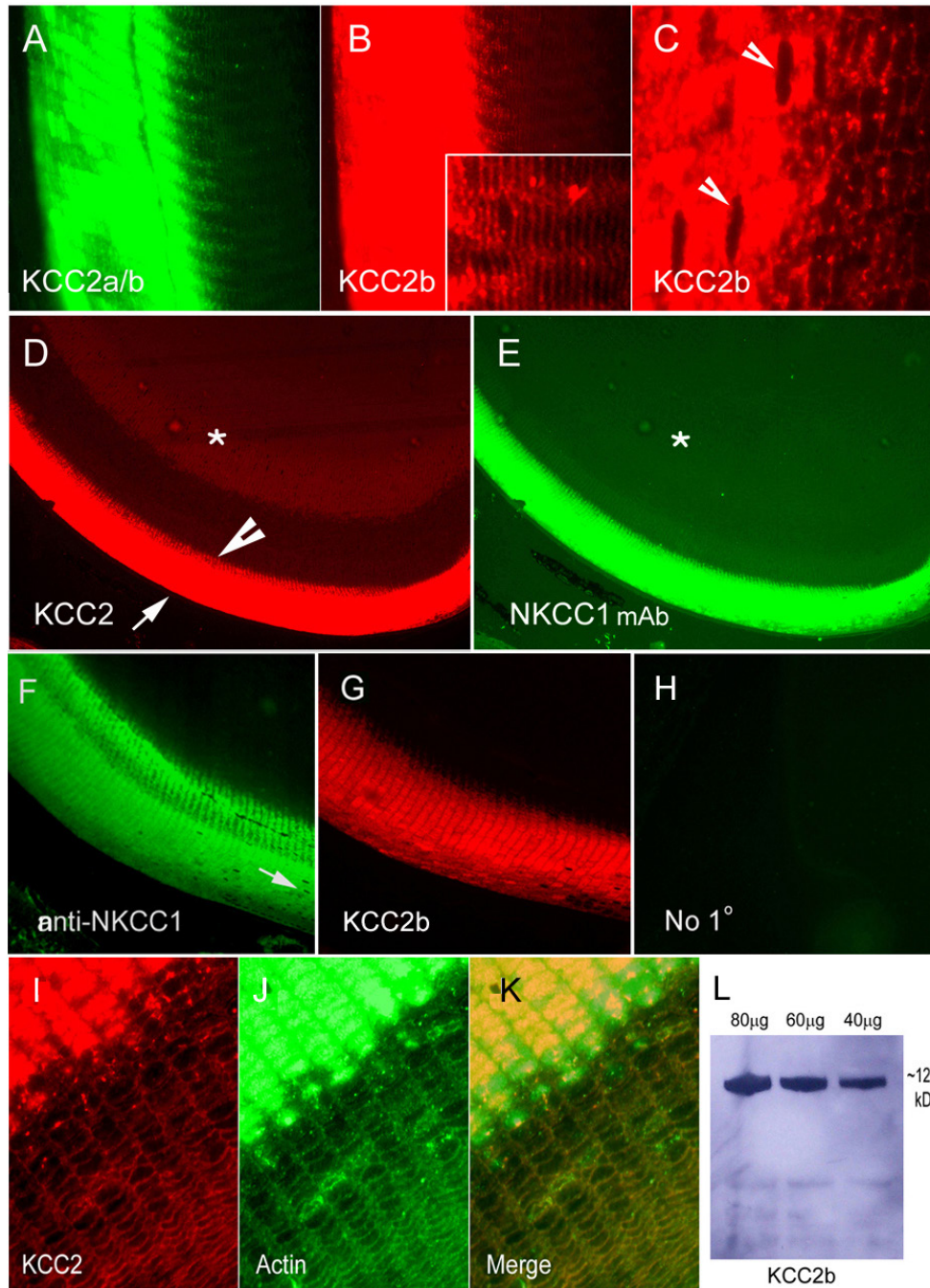


Figure 1. KCC2 and NKCC1 expression in a 30-day-old mouse lens. **A:** KCC2 mAb immunofluorescence detection (400X magnification) in mouse lens corresponds to the arrowhead in panel **D**. The lens perimeter is at the left. **B:** Anti-KCC2b (400X magnification; inset plus ~ 2X photomagnification). KCC2 is detected at the fiber cell borders in the interior lens fiber cells in the inset photo. Mouse lens fiber cells are about 7 µm in their longest dimension. **C:** Anti-KCC2b (600×, 3X photomagnification) identifies expression in cortical fiber cells as they terminally differentiate to mature fiber cells (arrowheads indicate the sites of the unstained cell nuclei). **D:** KCC2 is detected in interior fiber cells in the mouse lens (100X). The asterisk identifies the corresponding position in panel **E** and identifies KCC2 expression in the lens interior. The arrow at the lower left indicates the lens perimeter. **E:** NKCC1 mAb (monoclonal Ab) detection of cortical fiber cells with little detection in the interior lens nucleus. **F:** Lower exposure photo showing anti-NKCC1 polyclonal antibody detection restricted to lens cortical fibers in the mouse lens (200X, original magnification). **G:** KCC2b lower exposure detects protein in the cortical fiber cells. **H:** No primary antibody control. **I:** KCC2 detected at a site corresponding to the arrowhead in panel **D**. **J:** Actin detected in the same view as in panel **H**. **K:** **I, J** merged images. **L:** Immunoblot identifies KCC2b expression in the total mouse lens protein samples.

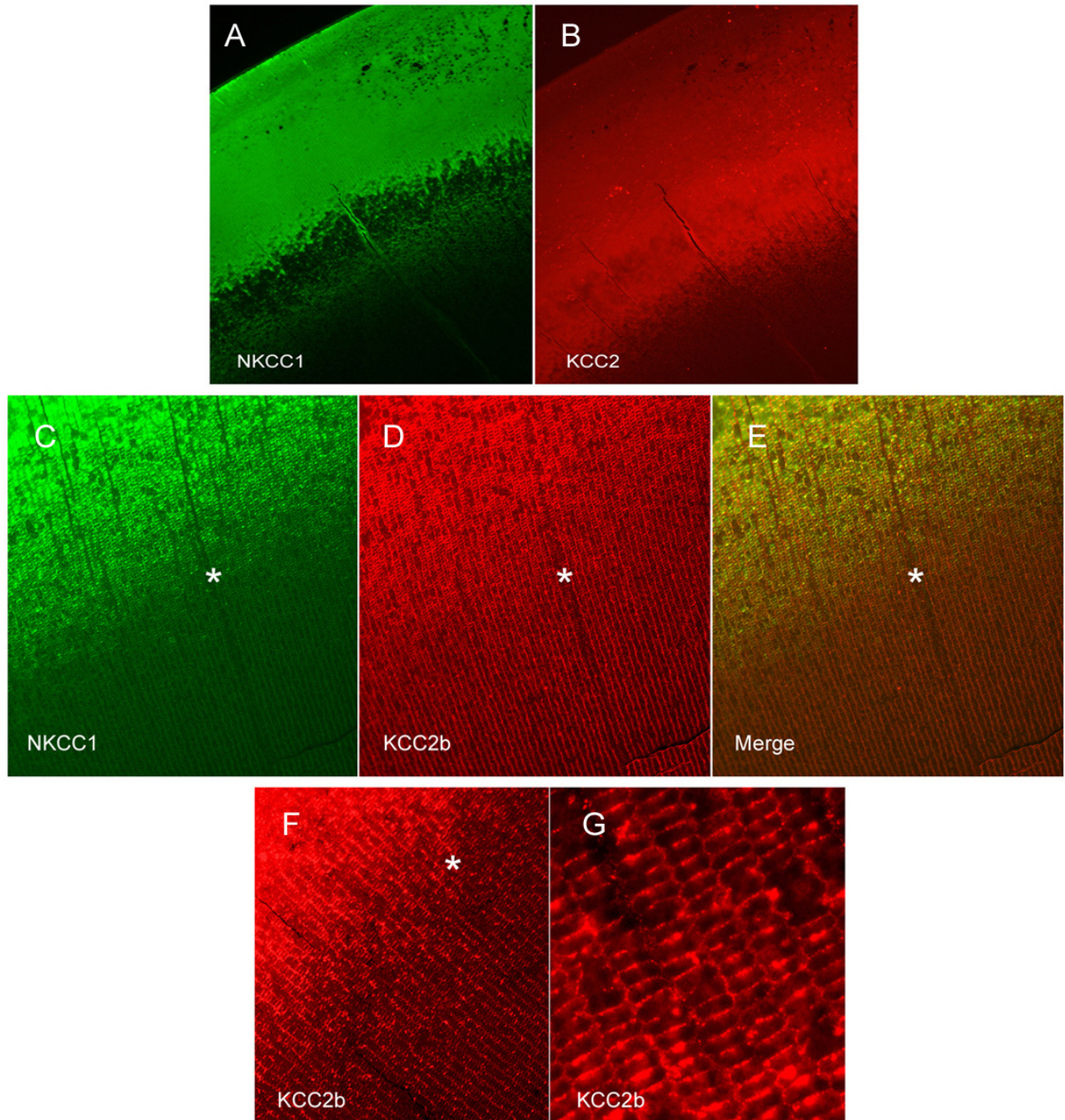


Figure 2. NKCC1 and KCC2b in situ immunofluorescence detection in young adult rabbit lens. **A, B:** NKCC1 mAb and anti-KCC2 antibody detection in young adult rabbit lens (100X). **C–E:** NKCC1 expression in the cortical fiber cells is decreased as fiber cells mature in the lens interior. KCC2 is detected in fiber cells closer to the lens center. Merged images **C, D** are shown in panel **E** (200X). **F:** KCC2b is detected at the fiber cell membrane borders in mature interior fiber cells (200X). **G:** KCC2b viewed at 600X magnification. Rabbit fiber cells are about 10 μm across in their longest dimension. The asterisks identify comparable positions in the panels.

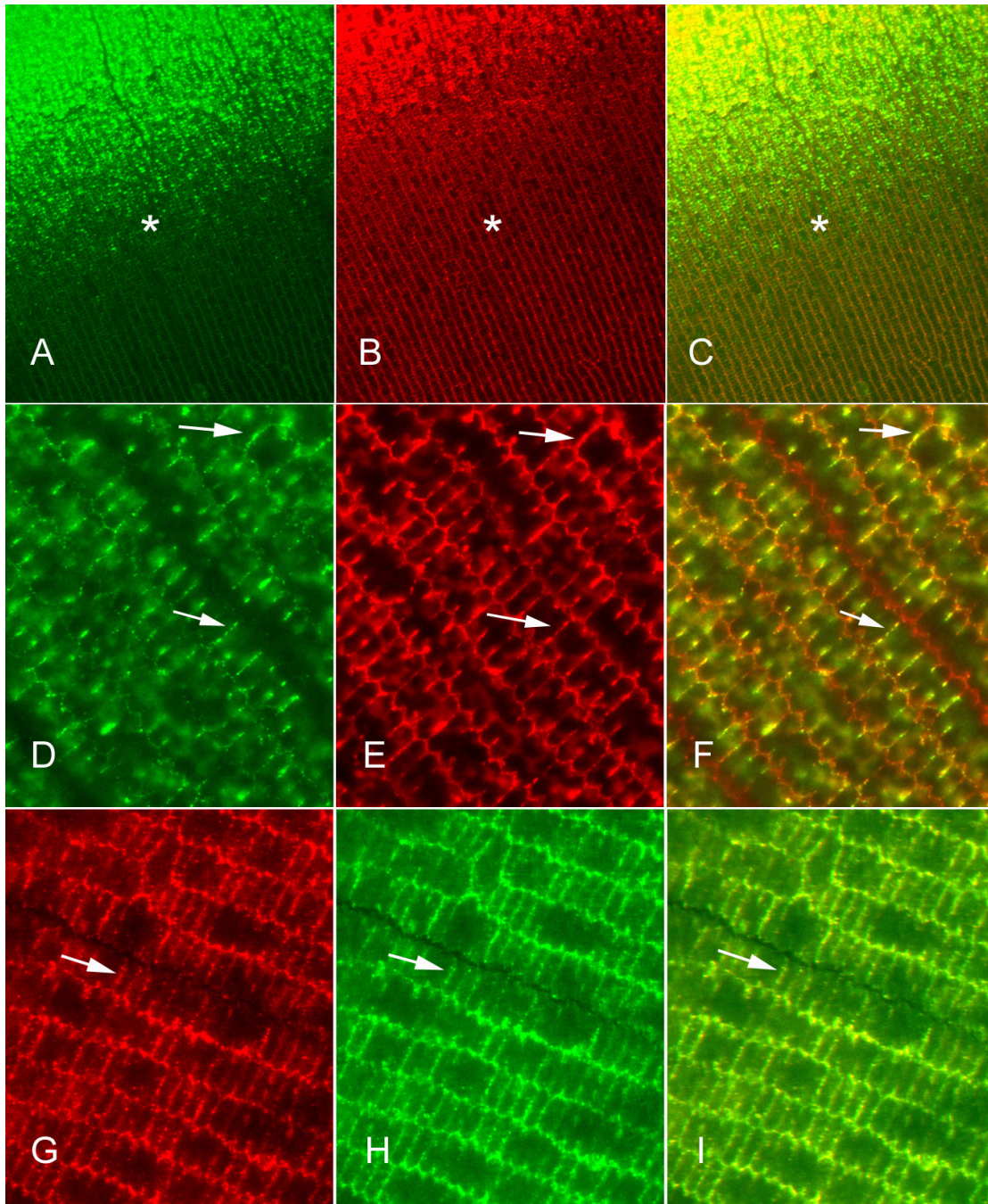


Figure 3. Detection of KCC2b, STEP, and GABA β 3 receptor subunit proteins in the young adult rabbit lens. **A–C**: Striatal enriched tyrosine phosphatase (STEP) mAb (green) and anti-KCC2b (red) immunofluorescence signals, with the merged images shown in panel **C** (200X). **D–F**: STEP (green) and KCC2b are present at the fiber cell borders in the fiber cells indicated by the asterisk in the panels above (600X, rabbit fiber cells are about 10 μ m across in the longest dimension shown). Arrows identify corresponding site in panels **D–F**. Panels **G–I** demonstrate KCC2b expression and GABA β 3 receptor subunit (green) in mature fiber cells corresponding to the site indicated by the asterisk in panels above.

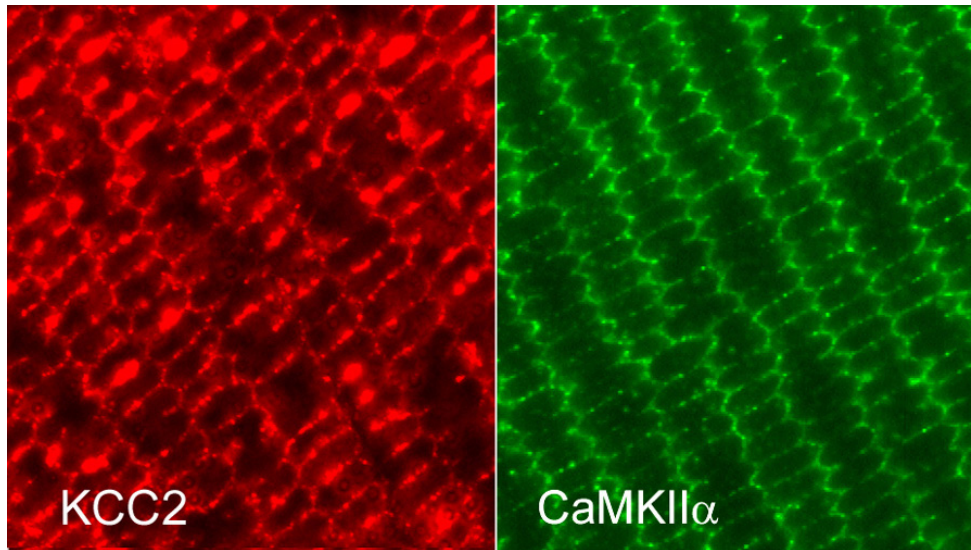


Figure 4. KCC2 and CaMKII α expression at the membrane borders between mature terminally differentiated fiber cells in the interior of the young adult rabbit lens. In the left panel, anti-KCC2 detects cross-reacting protein at fiber borders of mature fiber cells (corresponds to the asterisk in Figure 3) and similar to calcium calmodulin dependent kinase II α (CaMKII α) detection in the adjacent field of view in the right panel, consistent with findings from previous studies (24; rabbit fiber cells are about 10 μ m across in their longest dimension).

periphery may play a role in the cessation of DNA synthesis in differentiating fiber cells and contributes to a shift in growth factor actions from growth promoting to directing fiber cell differentiation as lens epithelial cells at the lens surface exit the cycle and begin to elongate and move into the lens. These observations suggest fiber cell elongation and neuron process formation may be similarly determined by the same battery of genes cited above. Findings that KCC2 in interior fiber cells is predominantly associated with fiber cell membranes suggest that KCC2's moonlighting structural roles interacting with fiber cell protrusion structural components may also apply in the lens.

Developmental regulation of NKCC1, KCC2, GABA synthesized by GAD25/65/67 and GAT proteins, AMPA/NMDA glutamate receptors, and an array of accessory signaling proteins are the principal determinants of neuron differentiation and are expressed in the lens. The extent this fundamental genetic regulon is conserved in the lens and expressed at comparable sites in lens cells provides evidence for a default hypothesis that this system functions in a comparable manner in lens development. However, we speculate that more dynamic activities that characterize neuronal and neural system signaling may have no direct parallels in the lens. Studies of rapid responses in neurons that can occur in milliseconds include the use of physiologically responsive dyes and electrophysiology measurements but may not be as relevant to the function of these proteins in lens morphogenesis. We hypothesize that the lens employs only ostensibly longer time-frame activities associated with these factors involved in cell growth and differentiation and is focused on the precise fiber cell growth required to produce an optically

useful tissue. However, the detailed conservation of this fundamental regulon in lens cells and neurons may suggest a role in coordinating the growth of lens cells and neurons to achieve vision acuity.

Visual acuity involves the coordinated growth of eye tissues and is guided by visual experience that includes circadian light cycles [22,35,36]. Melatonin has an important role in circadian regulation and is synthesized in the avascular lens by the same enzymes as in the pineal gland [37,38]. Melatonin synthesis and melatonin receptors are concentrated in peripheral lens fiber cells [37-39], and perhaps unexpectedly, melatonin levels were found to cycle up threefold each night in the lens similar to the brain [37,38]. Melatonin limits cell chloride efflux [40]. We speculate that increased melatonin at night may act synergistically with NKCC1 and further antagonize KCC2 to increase cell chloride and promote fiber cell differentiation at the expense of maturation at night. These findings can also suggest circadian rhythms in lens cells and neurons contribute to their coordinate regulation that contributes to achieving visual acuity.

KCC2 gene disruption identified no cytoarchitectural changes in the brain or cited in the eye [41]. These findings may relate to KCC2 homologs that provide redundancy. KCC2 knockout was linked with seizures and respiration defects at birth, similar to other synaptic protein genes that are also expressed in the lens including *PSD-95*, *CaMKII α* , and AMPA and NMDA glutamate receptor subunit genes [20,24]. Second, similar to KCC2, these membrane proteins are upregulated after birth in neurons after considerable brain and eye development has occurred, and additional studies can

determine whether these developmental patterns of expression are matched in the lens.

The present study provided evidence that developmentally regulated NKCC1 expression is restricted to peripheral actively differentiating fiber cells and that KCC2 predominates as fiber cells undergo maturation in the lens interior. Future studies can determine if NKCC1 expression is linked with increased fiber cell chloride and promotes fiber cell differentiation, and increased KCC2 as fiber cells mature is linked with cell chloride extrusion in a manner comparable with their antagonistic roles in neural development. Developmental delay in NKCC1 downregulated expression in neurons is considered key in neurodevelopmental disorders that include fragile X syndrome (FXS) and was demonstrated in the *Fmr1* knockout mouse model [3]. *Fmr1* gene mutations result in fragile X mental retardation protein (FMRP) depletion and underlie this disorder. *Fmr1* also has a basic role in eye and lens development [42,43] and can suggest that related effects on NKCC1:KCC2 developmentally regulated expression in response to *Fmr1* mutation/FMRP depletion has parallel effects in the lens.

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