PTBP-dependent PSD-95 and CamKIIα alternative splicing in the lens

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Purpose: Parallels described between neurons and lens fiber cells include detailed similarities in sub-cellular structures that increasingly show shared expression of genes involved in the construction and function of these structures in neurons. Intriguingly, associated modes of molecular regulation of these genes that had been thought to distinguish neurons have been identified in the lens as well. Both elongated cell types form membrane protrusions with similar size, shape, and spacing that exclude microtubules, contain F-actin, and are coated with the clathrin/AP-2 adaptor. Lenses express glutamate and gamma-aminobutyric acid (GABA) receptors with signaling and channel proteins shown to act together at neuronal membranes. Postsynaptic density protein 95 (PSD-95) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKIIα) expression and functions illustrate the integration of aspects of neuronal molecular and cell biology and were investigated here in the lens.
Methods: Immunofluorescence, immunoblot, and RT–PCR methods were used to assess protein expression and alternative transcript splicing.
Results: We showed the essential dendritic spine scaffold protein PSD-95 is expressed in lenses and demonstrated lens PSD-95 transcripts undergo polypyrimidine tract binding protein (PTBP)-dependent alternative splicing of its pivotal exon 18 required to avoid nonsense-mediated decay, and showed PTBP-dependent alternative splicing of CaMKIIα transcripts in the lens. The PSD-95 protein was observed at fiber cell membranes overlapping with N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate and GABA receptor proteins, tyrosine phosphatase STEP, CaMKIIα, the Ca(V)1.3 calcium channel, and clathrin, which were previously identified at lens fiber cell membranes. During neurogenesis, miR-124 is expressed that suppresses PTBP1 and promotes these splicing events. miR-124 is also expressed in mammalian lenses and upregulated during lens regeneration in amphibians, consistent with previous demonstrations of PTBP1,2 and PTBP-dependent PTBP2 exon 10 splicing in rodent lenses.
Conclusions: Findings of this dendritic spine scaffold protein and conservation of its key mode of molecular regulation in the lens provides further evidence that key aspects of the neuron morphogenetic program are shared with the lens.

Elongated lens fiber cells and neurons produce microtubule-based membrane vesicle transport systems along their length with similar ultrastructure [1] that include expression of RE-1 silencing transcription factor, neuron restrictive silencing factor (REST/NRSF)-regulated βIII-tubulin, synapsins, and other synaptic vesicle transport proteins [2-5]. Both cell types also produce arrays of membrane protrusions along the cells' lateral surfaces with similar size, shape, and spacing [6] that also express a similar set of proteins first linked with neuronal membranes and dendritic spines. We showed previously that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors expressed in adult lens fiber cells include the major neuronal subunits GluA1 and REST-regulated GluA2, NR1, NR2A, and NR2B [7-9]. Szabo and coworkers showed that 13 GABA receptor subunits and GABA metabolic enzymes are expressed in the lens beginning early in embryonic

lens development [10,11] and matched their expression profile in neural development [12]. Additional regulatory mechanisms shared in the lens include predominant Q/R RNA editing of lens GluA2 transcripts considered crucial in neurons [8]. At the protein level, lens GluA2 and NR2B are Tyr-phosphorylated at the clathrin/AP-2 adaptor interaction sites that promote GluA2/AMPAR and NR2B/NMDAR membrane insertion [7,8,13,14]. Consistent with this, lens and neuron membrane structures are coated with clathrin and adaptor protein AP-2 [6]. Tyrosine phosphatase STEP is also expressed in lens that acts on p-Tyr-GluA2 and p-Tyr-NR2B to promote AMPA and NMDA glutamate receptor internalization, and regulation of these p-Tyr modifications by STEP is a primary determinant of glutamate receptor membrane distribution in neuronal health and disease [7,8,13-15]. Glutamate and gamma-aminobutyric acid (GABA) receptors work with Ca^{2+}/calmodulin-dependent protein kinase (CaMKIIα) [2,16] and Ca(V)1.2 and 1.3 channel protein isoforms that are also present in fiber cells [2,7,8,10,17]. Moreover, both membrane structures are enriched in F-actin [6], which acts

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a scaffold in dendritic spines that determines spine size and shape, and underlies its functions [14,18,19].

Postsynaptic density protein 95 (PSD-95) is an essential scaffold protein just below the dendritic spine surfaces that organizes interactions of receptors, signaling proteins, and the F-actin meshwork beneath [14,18-22]. Additional factors include the ephrin A5 receptor also demonstrated at fiber cell membranes [23], which helps coordinate NMDA receptor and PSD-95 expression in neurons [24]. These unanticipated parallels in ultrastructure, constituent proteins, and molecular regulation suggested PSD-95 is also expressed in the lens. Lens protrusions have been described as forming ball-and-socket connections with adjacent cells. However, their roles in fiber cell morphogenesis or function are not yet well understood.

Neuronal PSD-95 expression requires a set of molecular factors that integrate control at the DNA, RNA, and protein levels and were first described as the principal determinants of the neuronal phenotype [2,3,9,25-29]. One component is the post-transcriptional regulation of alternative transcript splicing by PTBP RNA binding proteins [2,9,22,27]. PTBP1 is expressed in non-neuronal cells, neural progenitors, and lens epithelial cells [2]. As neurogenesis initiates REST-regulated miR-124 is expressed and suppresses PTBP1 [9,25,29,30]. miR-124 is also expressed in mammalian lenses [2,31] and was shown to be upregulated fourfold as lens regeneration is initiated in newts [32]. PTBP1 binding at its ribonucleotide recognition sites in primary transcripts suppresses the inclusion of adjacent exons [26]. PSD-95 exon 17–19 splicing encodes a premature nonsense stop codon that causes these transcripts to be degraded by the process of nonsense-mediated decay (NMD), and was described as ensuring neuron-specific PSD-95 expression [20,22]. Previously, we demonstrated the alternative splicing of PTBP2 exon 10 in lenses, which undergoes analogous miR-124, PTBP1 and NMD regulation, and showed PTBP-dependent splicing of neuronal C-src N1 exon in lenses [2,33]. Additional examples of PTBP-mediated alternative splicing verified in neurons include a CaMKIIα variable exon, although this splicing event is not required to avoid transcript degradation via NMD [27]. CaMKIIα and PSD-95 are among the most abundant dendritic spine proteins [20]. CaMKIIα has well-studied interactions with AMPA, NMDA, and GABA receptors, channels, and Ephrin receptors in neurons [16,21] and is expressed in the lens [2]. PSD-95 interactions with the F-actin meshwork further emphasize the organizational role of this protein at membrane spines [18,19,22]. Here, we determined PSD-95 and CaMKIIα expression and alternative splicing in the lens.

METHODS

Animals: Lenses of wild-type New Zealand white rabbits about 3–5 months old were isolated from six rabbits obtained from Goffel Farms (Wyckoff, NJ) or were purchased from Pell-Freeze (Rogers, AR). 28-day-old BalbC mouse lens tissues were a gift of the Rutgers BHS animal care facility, originally obtained from Charles River Laboratories (Charleston, NC). Animals were treated in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and the protocol was approved by Rutgers IACUC.

Immunological techniques: In vitro biochemical experiments used lenses homogenized in radioimmunoassay (RIPA) buffer with 2% sodium dodecyl sulfate (SDS) and protease inhibitors (Sigma, St. Louis, MO). Subsequently, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the protein samples resolved on the immunoblots. The blots were probed with antibodies according to the manufacturer's recommendations. Immune complexes were visualized with chemiluminescence (Millipore, Billerica, MA).

Eyes and lenses prepared for histological examination in paraffin sections were fixed in buffered 4% paraformaldehyde. Indirect immunofluorescence studies used sections dewaxed in xylenes and graded alcohol washes. Sections blocked in TBS/5% serum for 1 h were incubated with primary antibodies (1:200) overnight at 4 °C. After washing, fluor-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were applied (1:200) in TBS/serum for 1 h. Antibodies included anti-PSD-95 (Genscript, Piscataway, NJ), tyrosine phosphatase STEP mAb (Santa Cruz, Paso Robles, CA; Cell Signaling Beverly, MA), anti-PTBP2 (gift from A. Willis, Leicester, UK), anti-GABA(A) β3 (Santa Cruz), anti-clathrin and anti-CaMKIIα (Sigma), anti-Ca(V)1.3 (Neuromab, Davis, CA), and anti-C-terminal-p-Tyr-GluA2 (C-terminal) tyrosine and anti-p-Tyr-NR2B (tyrosine-1472; Cell Signaling).

RNA analysis: Analysis of the alternatively spliced transcripts used RT–PCR kits (Qiagen, Germantown, MD). Lens tissue was extracted in TRI Reagent (Sigma) to purify the total RNAs. Reactions used pre-mixed reagents (One-Step RT-PCR kit, Qiagen) with 0.6 µM primers and 2 µg RNA, with sequential incubations as follows: 50 °C/30 min, 95 °C /15 min, 94 °C /15 min, 57 °C /30 s, 72 °C /1 min repeated for 32 cycles and included a final 10 min extension cycle. Exon-specific primers corresponded to PSD-95 5′-TCT GTG CGA GAG GTA GCA GA-3′ and 5′-AAG CAC TCC GTG AAC TCC TG-3′ and CaMKIIα: 5′-CGG AGG AAA CAA GAA GAA CG-3′ and exon 3: 5′-CCT CTG GTT CAA AGG CTG TC-3′. cDNA products resolved with electrophoresis were

purified for DNA sequencing (Rutgers Molecular Resource Facility, Newark, NJ).

RESULTS

Expression of PSD-95 protein in the lens: PSD-95 expression and functions in neurons illustrate the integration of basic aspects of neuronal molecular and cell biology. We began our study by determining whether the PSD-95 protein is expressed in mammalian lenses. Immunoblots identified expression of PSD-95 in rabbit and mouse lenses (Figure 1A,B). We next confirmed that PTBP2 (nPTB) is expressed in rabbit lenses (Figure 1C), which agrees with our previous identification of PTBP2 in mouse lenses [9,33]. Expression of the PSD-95 protein indicated PSD-95 exon 18 alternative splicing occurs in the lens, consistent with the PTBP2 exon 10 alternative splicing we showed previously in lenses [33], which is also necessary to produce a coherent full-length transcript for productive protein translation [22]. Lenses are comprised of cuboidal lens epithelial cells that cover their anterior surface. At the anterior/posterior equator, these cells exit the cell cycle and begin a process of pronounced elongation as they move into the lens interior. Examination of PTBP2 protein distribution in rabbit and mouse lens sections (Figure 1D) identified PTBP2 in peripheral differentiating fiber cells (lens cortex) in rabbit and mouse lenses, with less signal in mitotic lens epithelial cells below the lens capsule at the anterior surface, or terminally differentiated mature fiber cells in the center of the lens in the interior (lens nucleus), and agreed with our earlier examination of mouse lenses [2,9].

PSD-95 protein distribution at fiber cell membranes: We next examined PSD-95 protein distribution in rabbit and mouse lenses in situ in histological sections. We observed the PSD-95 protein is largely detected at the borders of fiber cells in mouse and rabbit lenses (Figure 2). We next compared PSD-95 spatial expression in lenses with that of glutamate and GABA receptor proteins and additional signaling proteins shown previously to localize at neuronal membranes and dendritic spines and at fiber cell membranes in the lens. PSD-95 was observed at the fiber cell perimeter and overlapped with lens detection of the GABA(A) β3 receptor subunit [10], tyrosine phosphatase STEP [8], CaMKIIα [2], p-Tyr-NR2B [7], and Ca(V)1.3 L-type calcium channel expression [17], as well as clathrin [6] at fiber cell borders (Figure 2). Our examination of lenses also showed comparable distributions of PSD-95 and C-terminus-Tyr-phosphorylated GluA2 at fiber cell borders (Figure 3). Enlargement of photomicrographs indicated the punctate nature of staining along fiber cell perimeters consistent with focal concentrations of these proteins at the fiber cell borders,
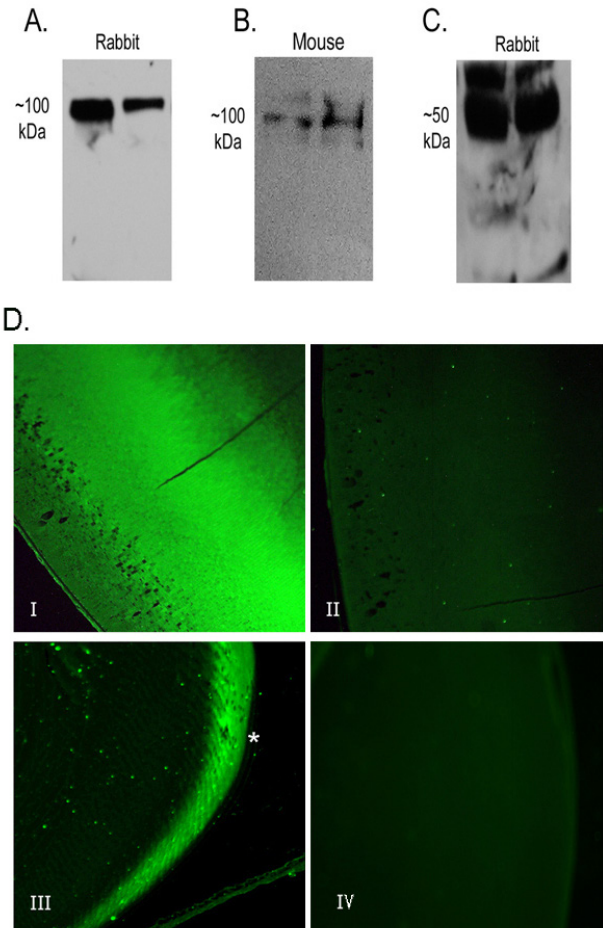

Figure 1. Post-synaptic protein 95 (PSD-95) and polypyrimidine PTBP2 (nPTB) proteins are expressed in approximately 4-month-old rabbit and 28-day-old mouse lenses. **A**: Immunoblot detection of about 100 kDa PSD-95 in two individual rabbit lens samples. **B**: Detection of PSD-95 in two mouse lens samples of four lenses. **C**: Detection of about 50 kDa PTBP2 (nPTB) in rabbit lens. **D**: In situ detection of PTBP2 in rabbit and mouse lens. Photos show peripheral lens fibers at the lens border: I) indirect immunofluorescence detecting PTBP2 in rabbit lens, II) no primary antibody control in rabbit lens, III) anti-PTBP2 mouse lens, and IV) no primary control in mouse lens (1:200 original magnification; asterisk adjacent to lens epithelial cells beneath capsule at the anterior surface).

similar to our previous study of p-Tyr-NR2B in the lens [7] (Figure 3). However, ultrastructure studies can determine specific associations with adjacent or proximal membranes and at membrane protrusions in the lens in greater detail, similar to dendritic spines.

PTBP dependent alternative splicing of PSD-95 and CaMKIIα transcripts in lens: We next examined PSD-95 expression at the RNA level in the lenses of 1-month-old mice to determine if PSD-95 exon 18 splicing occurs in the lens. Total lens RNA samples were used to produce cDNAs amplified with
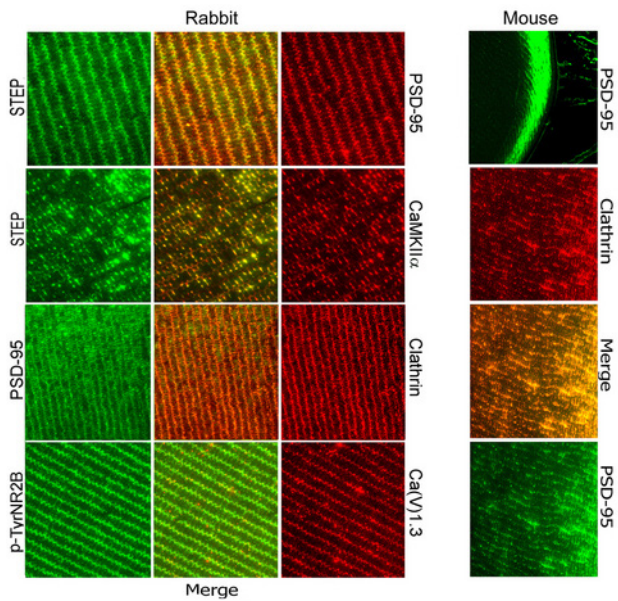
Figure 2. In situ indirect immunofluorescence detects PSD-95 in about 4-month-old rabbit and 28-day-old mouse lenses at the fiber cell borders, overlapping with additional neuronal membrane markers shown previously to be expressed at lens fiber cell membranes. Left panels: IF detection of PSD-95, tyrosine phosphatase STEP, CaMKIIα, clathrin, P-Tyr-NR2B, and Ca(V)l.3 in the rabbit lens histological sections (original magnification 600X). Merged views show dual fluorescence detection demonstrated in adjacent panels. Right panels: The top panel shows detection of PSD-95 in the mouse lens (original magnification: 200X). The three panels below demonstrate dual fluorescence detection of PSD-95 and clathrin in the mouse lens (original magnification: 600X). Negative controls with primary antibody are omitted since they appeared in Figure 1.

exon-specific primers. Amplification of PSD-95 transcripts identified a major product of expected size from lens RNA (Figure 4A). The nucleotide sequence determined for this product corresponded to PSD-95 in the GenBank database, and showed exon 18 is included in lenses with expected exon junctions shown in nucleotide sequence histograms (Figure 4A). Our examination of an additional PTBP-dependent alternative transcript splicing verified first in neurons involves the alternative primary transcript splicing of CaMKIIα in its variable exon region [26,27]. Examination of RNAs from 1-month-old mouse lenses with RT–PCR produced a major CaMKIIα cDNA product that when sequenced identified variable exon 15 in CaMKIIα transcripts in the lens, and matched transcripts produced in neurons [27] (Figure 4B).
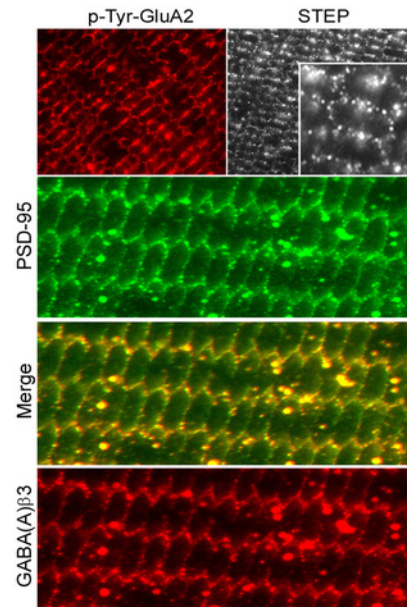

Figure 3. Fluorescent antibody detection of p-Tyr-GluA2, STEP Tyr-phosphatase, PSD-95, and GABA(A)β3 receptor subunits in an approximately 4-month-old rabbit lens. The top two panels show detection of C-terminus-p-Tyr-GluA2 in the color image (left) and detection of tyrosine phosphatase showing grayscale intensity for Alex 488 detection. The inset photo shows about 2.5-fold magnification. The three panels below show detection of PSD-95 and GABA(A)b3 subunit in the same field in the rabbit lens (original magnification 600X and magnified about 2.5-fold).

DISCUSSION

Our findings showed the essential dendritic spine scaffold protein PSD-95 is expressed in lenses and localizes at the fiber cell membranes together with a similar group of glutamate and GABA receptor, CaMKIIα, clathrin, calcium channels, and STEP proteins, which are also found at neuronal membranes and with interdependent functions at dendritic spines [6–10,23,33]. A second key finding from our study showed that lens PSD-95 transcripts include alternatively spliced exon 18, which is consistent with productive PSD-95 protein translation in lenses. The identification of this specific RNA cleavage and re-joining reaction in lens was previously characterized as neuron-specific and may suggest that additional factors involved in this process in neurons are present in the lens as well. The present findings agree with miR-124 expression and suppression of PTBP1, as well demonstrations that PTBP2 exon 10 and Neuronal C-src N1 exon alternative splicing also occur in the lens [2,9,31,32]. Together with the present demonstration of CaMKIIα alternative exon splicing in the lens, these findings appear consistent with a related alternative splicing mechanism involving miR-124 and PTBPs operating in the lens. In addition, similarities in the F-actin meshwork described in fiber cell and neuron
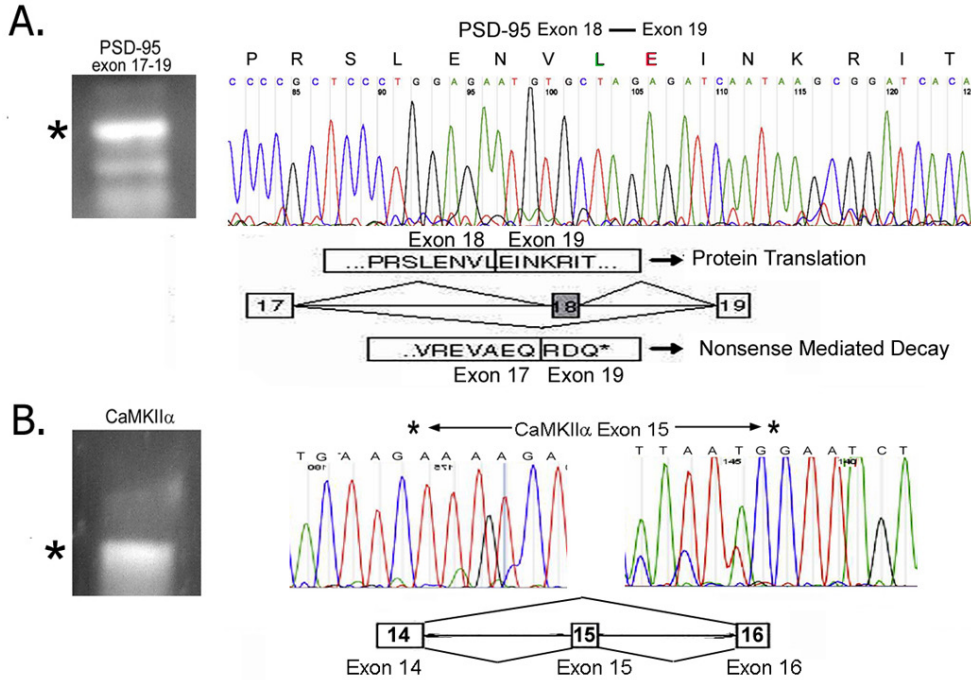
Figure 4. RT–PCR assay of transcripts present in 28-day-old mouse lenses. Amplified cDNA products corresponding to PSD-95 exon 17–18–19 and CaMKIIα variable exon 15 are shown. **A**: cDNA product resolved with electrophoresis using PSD-95 exon-specific primers. The adjacent nucleotide histogram shows the nucleotide sequence demonstrating the exon 18–19 splice junction and the corresponding amino acid sequence. **B**: cDNA product resolved with electrophoresis corresponding to the CaMKIIα transcripts amplified from the variable region. The adjacent nucleotide histogram shows sequences corresponding to the upstream and downstream exon for exon 15.

membrane protrusions [6] lead us to speculate that PSD-95 interactions with the dendritic spine cytoskeleton may also have parallels in the lens.

The human *Psd-95* gene has been linked with neuropsychiatric disorders [20,34]. PSD-95 belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins [20], and the gene is located on human chromosome 17 at 17p13.1 near the D17S796 marker [35]. We note that a locus for bilateral autosomal dominant anterior polar cataract was identified near this marker [36], and future studies can determine if PSD-95 might be involved. In contrast, the PSD-95 knockout mouse is similar to gene disruptions of synapsins, other synaptic vesicle transport genes, glutamate and GABA receptor genes, and STEP, which produced no cytoarchitectural changes in the brain [34]. These findings have been attributed to compensation during development due to gene redundancy similar to other tissues [37]. In addition, CaMKIIα and PSD-95 do not show significant expression in neurons until several days after birth, which has suggested their functions are associated with neuron maturation, and may suggest a similar expression profile during lens development [22,27,38].

NMDA receptor membrane localization has also been linked with the receptor's coordinated interactions with Cdk5 and Neuronal C-src kinase and PSD-95 [39], which are also expressed in the lens [2,40]. Tyrosine 1472 phosphorylation of NR2B at its clathrin/AP2 interaction site blocks receptor endocytosis, and p-Tyr-NR2B also occurs in the lens [7]. In neurons, Cdk5 facilitates p-Tyr1472 phosphorylation by increasing neuronal C-src interaction with NR2B mediated by the PSD-95 scaffolding protein [39]. Conversely, Tyr-phosphatase STEP uncovers these sites allowing clathrin/AP2 interaction and receptor internalization [13].

Evidence of additional modes of neuron-like alternative splicing in the lens was seen in our earlier demonstrations that "brain-specific" Fox-1 RNA binding protein alternatively spliced isoforms are produced in lenses [33] and that Fox-1 associated NMDA receptor NR1 exon 5 splicing occurs in the lens as well [7]. Our previous studies demonstrated neuron-like splicing of neurofibromatosis 1, CGRP, and neuronal cell adhesion molecule 180 transcripts in lenses [2,41]. Last, Alzheimer Aβ precursor protein alternative splicing that produces neuronal AβPP 695 transcripts was shown to be dependent on miR-124 expression [42], and this splicing event also occurs in the lens [43].

The role of miR-124 suppression of PTBP1 transcripts is also dependent on the reciprocal relationship of miR-124 with the REST transcription factor [25]. miR-124 gene expression is suppressed by REST in non-neuronal cells (although lenses were not examined). As neurogenesis is initiated, REST levels decrease, and miR-124 is expressed. miR-124 also binds REST transcripts to ensure suppression in post-mitotic neurons. Studies to date showed that REST decreases as fiber cell elongation proceeds and that miR-124

is expressed in mammalian lenses. In addition, miR-124 substantially upregulates during lens regeneration in amphibians. miR-124 suppresses expression of about 200 transcripts and was also shown to be neuron specific in a 44 tissue gene array study that also did not include the lens [44]. However, miR-124 distribution within the lens has not yet been determined, although Dicer miRNA processing enzyme has been observed in fiber cells.

Neuronal development has been described as one of the most protracted cell differentiation processes [27] and is matched by cells in the lens. Analysis of PTBP expression and its transcript targets identified three stages of neuron differentiation: mitotic progenitors, differentiating neurons, and mature neurons, which can be defined by inverse levels of PTBP1/PTBP2 expression as neurogenesis proceeds [27]. Many genes linked with dendritic spine biology do not show expression until post-natal development and include PTBP associated PSD-95 and CaMKIIα expressed first at post-natal day 5 in brain [22,27,38,45]. Future studies can determine if a similar relationship exists among lens epithelial cells, differentiating cortical fiber cells, and terminally differentiated nuclear fiber cells.

ACKNOWLEDGMENTS

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