Published in final edited form as:

Brain Res. 2008 June 18; 1215: 20–29. doi:10.1016/j.brainres.2008.03.038.

COMPARISON OF THE ONTOGENY OF THE VESICULAR GLUTAMATE TRANSPORTER 3 (VGLUT3) WITH VGLUT1 AND VGLUT2 IN THE RAT RETINA

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Abstract

Glutamate is the major excitatory neurotransmitter in the retina, and most glutamatergic neurons express one of the three known vesicular glutamate transporters (VGLUT1, 2, or 3). However, the expression profiles of these transporters vary greatly in the retina. VGLUT1 is expressed by photoreceptor and bipolar cell terminals, and VGLUT2 appears to be predominately expressed by ganglion cells, and perhaps Müller cells, cone photoreceptor terminals, and horizontal cells in some species. The discovery of a third vesicular glutamate transporter, VGLUT3, has brought about speculation concerning its role and function based on its expression in amacrine cells. To address this we studied the postnatal development of VGLUT3 from day 0 through adult in the rat retina, and compared this with the expression patterns of VGLUT1 and VGLUT2. VGLUT3 expression was restricted to a population of amacrine cells. Expression of VGLUT3 was first observed at postnatal day 10 (P10) in the soma and some processes, which extensively arborized in both the ON and OFF sublamina of the IPL by P15. In contrast, VGLUT1 and VGLUT2 expression appeared earlier than VGLUT3; with VGLUT1 initially detected at P5 in photoreceptor terminals and P6 in bipolar terminals, and VGLUT2 immunoreactivity initially detected at P0 in ganglion cell bodies, and remained prominent throughout all stages of development. Interestingly, VGLUT3 has extensive somatic expression throughout development, which could be involved in non-synaptic modulation by glutamate in developing retina, and could influence trophic and extra-synaptic neuronal signaling by glutamate in the inner retina.

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Keywords

VGLUT1; VGLUT2; ribbon synapse; amacrine cell; glycine

INTRODUCTION

Neurotransmitters are the key information molecules of the retina, and they mediate both excitatory and inhibitory neural signals. L-glutamate has been established as the fast excitatory neurotransmitter in the vertebrate retina [47], and it defines the excitatory vertical throughput pathway in the retina, from photoreceptors in the outer retina to ganglion cells in the inner retina. All glutamatergic neurons are known to express at least one of the three types of vesicular glutamate transporters (VGLUT1, VGLUT2, and VGLUT3) [2,10–12,42,45,46,51]. These transporters are members of brain-specific Na⁺-dependent inorganic phosphate cotransporter family, which was originally thought to be a carrier for inorganic phosphate, but acted as a vesicular glutamate transporter [2]. These transporters share roughly 70% amino acid homology among each of the VGLUTs and likely have similar structural topology which includes 8 to 10 putative transmembrane regions [42]. VGLUT activity is dependent on the vesicular proton electrochemical gradient generated by the vesicular proton ATPase, and a unique dependence on Cl[−], which serves as a counter ion of H⁺ influx mediated by the ATPase [2,42]. The pharmacology, substrate specificity and kinetics are similar among all three known VGLUTs [42].

In contrast to these similarities, the three VGLUTs differ in their expression profiles in both the retina and central nervous system [10,11,16,21,24,25,32,38,41,42,50]. In the adult retina, VGLUT1 is expressed predominately in the two synaptic layers containing ribbon synapses: the outer plexiform layer (OPL) and inner plexiform layer (IPL) [25,41]. VGLUT2 is expressed in cone photoreceptors [14,50] and ganglion cell bodies and their dendrites within the ganglion cell layer (GCL) and the IPL [25,41,50], and perhaps some Müller cells [25,41], and horizontal cells [15]. VGLUT 3 selectively labels a subset of amacrine cells and their processes within the IPL that contain glycine and the glycine transporter 1 [21,24].

In the adult brain VGLUT1 and VGLUT2 display roughly complementary expression patterns. For example, VGLUT1 predominates in the cerebellum and hippocampus [5,12,22], whereas VGLUT2 expression is prominent in the spinal cord [26]. However, none of these brain regions exclusively express one isoform, and although the majority of glutamatergic neurons express either VGLUT1 or VGLUT2, several studies describe co-expression of both isoforms, for example, in neurons in the barrel cortex [27] and cerebellum [35]. Most remarkably, a developmental switch occurs from VGLUT2 to VGLUT1 in the cerebellum [5,33]. In contrast, in the developing rat retina, VGLUT1 and VGLUT2 are not co-expressed in photoreceptors and ganglion cells, and these transporter isoforms do not switch their expression between different cell types [25]. An exception to these findings is the co-expression of VGLUT1 and VGLUT2 in about 10% of the cone photoreceptor terminals in the mouse retina [50]. The developmental expression pattern of VGLUT3 has only been examined in mouse retina and it does not overlap with VGLUT1 or VGLUT2 expression [24].

The present study investigates the expression of VGLUT3 in the developing rat retina; postnatal retinas from postnatal day 0 (P0) to adult were immunolabeled with selective antibodies to VGLUT1, 2, and 3. The postnatal expression pattern of VGLUT3 was compared with the expression patterns of VGLUT1 and VGLUT2 in the rat retina. These findings show that VGLUT3 expression was first detected at P10 in the soma and some processes of a single population of amacrine cells. These VGLUT3 containing amacrine cell processes did not extensively arborize in the IPL until P15, where they formed extensive arborizations in both

the ON and OFF sublamina. This is in contrast to VGLUT1 expression, which appears at P5/6 and is restricted to ribbon synapses in the OPL and IPL. Faint VGLUT2 expression appears early in development at P0 in the GCL, and becomes stronger by P6. The expression profiles of each of the VGLUTs demonstrate that these glutamatergic synapses are not overlapping or redundant as revealed in the brain (i.e., there is no apparent switch from expression of any one VGLUT isoform to another at any synapse in the rat retina). Instead in the rat retina, VGLUT3 is restricted to a subset of amacrine cells during development, like mouse, suggesting that these synapses are unique from other glutamatergic synapses in the rat retina containing VGLUT1 or VGLUT2.

RESULTS

Distribution of the VGLUTs in the adult mammalian retina

VGLUT1 immunoreactivity was present in synaptic terminals of both bipolar cells in the IPL and photoreceptors in the OPL, respectively (Fig. 1A). VGLUT1 immunostaining in the IPL showed partitioning of bipolar cell terminals to both the ON and OFF sublamina of the IPL, and prominent immunostaining of the rod bipolar cell terminals in the ON sublamina of the IPL (Fig. 1A). A double label experiment with VGLUT1 and PKC α antibodies showed the expression of VGLUT1 in rod bipolar cell terminals (Fig. 1A–C). A double label experiment with CtBP2 and VGLUT1 antibodies showed that CtBP2, a marker of synaptic ribbons co-localized with VGLUT1 (Fig. 1D–E), confirming previous studies showing that VGLUT1 labels ribbon synapses of photoreceptors in the OPL [25,41]. VGLUT2 was found in the IPL of the retina as previously reported [25,41], but immunoreactivity was present in the cell bodies of the GCL and nerve fibers of these cells and faint immunoreactivity was observed in Müller cell processes (Fig. 1G). VGLUT2 immunoreactivity was not found in rat cone photoreceptor terminals as previously reported in mouse and cat retina or horizontal cells as reported in rat retina [14,15,50]. VGLUT3 is expressed by a subset of amacrine cells, with processes that stratify into two narrow regions within the ON and OFF sublamina [21,24]. This can be seen in the IPL with triple immunolabeling with VGLUT3, CtBP2 and CaBP5 (Fig. 1 H–K).

Ontogeny of VGLUT3 in the mammalian retina

The developmental expression of VGLUT3 showed a distinct temporal sequence (Fig. 2). Weak VGLUT3 labeling was first detected in developing amacrine cells in the proximal retina by P10, with the most intense immunoreactivity appearing in the primary process entering the IPL (Fig. 2E). Expression was also present throughout the soma at P10, and it increased at later developmental stages (P15–P25) (Fig. 2F–H). VGLUT3 immunostaining increased around P20 and it was localized to large arborizations and fine process within the IPL. It is not clear which bipolar cell or ganglion cell subtype forms synapses with VGLUT3 amacrine cell processes. However from the development of VGLUT1 in the IPL (see Fig. 3), it is clear that formation of these synapses by bipolar cells within the ON and OFF sublamina likely precede the synapse formation of VGLUT3 containing amacrine cells.

Developmental expression of VGLUT1 in ribbon synapses of mammalian retina

VGLUT1 was expressed at ribbon synapses in the OPL and IPL of the rat retina (Fig. 1A), and it was absent from all conventional synapses (amacrine-ganglion cell synapses) in rat and mouse retinas [25,41]. The developmental expression of VGLUT1 showed a distinct temporal sequence (Fig. 3). Weak VGLUT1 immunolabeling was first revealed in developing photoreceptor terminals within the OPL at P5 (Fig. 3B), corresponding to the ontogenesis of the cone photoreceptor synapse [4], and followed by development of glutamatergic signals in the IPL at P6 (Fig. 3C), which is correlated with the development of bipolar cell terminals in the OFF sublamina of the IPL [41]. In the OPL the levels of VGLUT1 immunostaining in photoreceptor terminals increased during the second postnatal week and at P15 were similar

to adult levels (Fig. 3G). In the IPL, the levels of VGLUT1 immunostaining appeared within both the ON and OFF sublamina by P10 (Fig. 3F, inset), immunostaining within both the OPL and IPL was prominent by P15 and afterward, characterized by strong immunoreactive puncta in these synaptic layers (Fig. 3H–I), which is identical to the expression observed in the adult retina (Fig. 1A).

Developmental expression of VGLUT2 in ganglion cells of mammalian retina

Unlike VGLUT1, VGLUT2 was expressed predominately in cell bodies of GCL (Fig. 4). This observation is in agreement with previous reports from other groups studying VGLUT2 in rodent retina [13,25,41]. Very faint immunoreactivity in the somas was first observed in the GCL at P0 (Fig 4), the expression increased at P6 and remained strong through to the adult retina. Faint immunoreactivity was also present in the IPL at P10 and remained present in the adult retina (Fig. 1G and Fig. 4). VGLUT2 immunoreactivity in Müller cells has been reported in mouse retina [25,41], however as suggested previously this immunostaining might be non-specific [25]. In addition, one study observed VGLUT2 immunolabeling in cone terminals of the mouse retina [50], and VGLUT2 immunolabeling in horizontal cells of the adult rat retina [15]. However, we observed no obvious labeling of VGLUT2 in the OPL suggesting that VGLUT2 expression is either absent from cones or horizontal cells in the rat retina, or the fixation conditions and/or tissue preparation might be the reason for the lack of signal in the OPL of the rat retina.

DISCUSSION

We evaluated the developmental expression pattern of VGLUT3 and compared that to the ontogeny of VGLUT 1 and 2 in the rat retina. The expression of VGLUTs in postnatal rat retina suggests that these proteins underlie distinct functions involving glutamate release. VGLUT1 was expressed solely in photoreceptor and bipolar cell terminals in rat retina during development (Fig. 3), suggesting that glutamate acts as a transmitter at ribbon synapses as previously reported [25,41]. VGLUT2 was expressed primarily in cell bodies within the GCL during development (Fig.4), this finding is consistent with the reported localization of VGLUT2 in glutamatergic neurons in the retina [13,25,41]. The distribution of VGLUT2 in cell bodies contrasts strongly with that of VGLUT1 in photoreceptors and bipolar cells because VGLUT1 was restricted solely to synaptic terminals during development (Fig. 3 and Fig. 4). However, the somatic distribution of VGLUT2 in the GCL is similar to the somatic distribution of VGLUT3 in amacrine cells. VGLUT3 is expressed in all cell compartments during development, the soma, axon and processes (Fig. 2); suggesting that these transporters may be involved in functions other than the synaptic release of glutamate.

In this study we found that the onset of VGLUT3 expression was late in rat retinal development, like in the mouse retina [24] and it appeared at P10. This is in contrast to the expression of VGLUT1 or VGLUT2, which appears as early as P5 and P0, respectively. VGLUT3 is expressed much later than VGLUT1 and VGLUT2, this late VGLUT3 expression suggests that synapse formation within the glutamatergic vertical throughput pathway precedes the development of VGLUT3 containing synapses in amacrine cells.

VGLUT3 was most likely in synaptic vesicles of amacrine cell terminals [10], but as mentioned before, VGLUT3 exhibits strong expression within non-synaptic structures of amacrine cells, including the soma, processes, and axons which all contain significant VGLUT3 immunoreactivity (Fig. 2, P10–25). This observation is in agreement with other reports on VGLUT3 expression in the retina [21,24]. VGLUT3 has also been associated with synaptic vesicles and vesicle-like structures in the somata and dendrites of a subset of neurons in the CNS [5,10], suggesting that VGLUT3 participates in non-synaptic glutamate release, like that observed in astrocytes and oligodendrocytes [1,3]. Ultrastructural electron microscopic studies

have also localized vesicle-like structures containing VGLUT3 in the cell bodies of granule cells in the cerebellum, which is consistent with the involvement of VGLUT3 in nonsynaptic modulation by glutamate [5,17]. Ectopic release of synaptic vesicles from glutamatergic neurons has been reported [31], and VGLUT3 has been implicated in retrograde signaling of neurons [19]. This form of perisomatic stimulation could modulate the synchronizing of the action potential firing in adjacent amacrine cells as described for granule cells in the cerebellum [43]. Furthermore, VGLUT3 in amacrine cells might mediate a developmental cue involving glutamate release that functions in a trophic capacity to maintain neuronal excitability. However, in light of this potential trophic role for VGLUT3, it must occur at a latter stage in development in the rat retina, since VGLUT3 expression is first observed at P10 (Fig.2E).

Our findings indicate that rat retinal VGLUT3 expression during development differs slightly from another study in mouse showing that VGLUT3 first appears at P7/8 (Johnson et al., 2004) versus P10 as observed in this study (Fig. 2E). Ruling out differences in tissue fixation, antibody penetration and tissue preparation it is possible that VGLUT3 expression might occur later in rat than mouse. This observation is consistent with a delay of 1.5 to 2 days for rat retina, compared to the same developmental stage for mouse retina (e.g., eye opening occurs at P12–14 in mouse and P13–15 in rat) [(source:

<http://embryology.med.unsw.edu.au/OtherEmb/Rat.htm>:[41]]. A delay in expression of VGLUT1 was also observed between mouse and rat, where VGLUT1 expression first appeared at P3 in mouse retina, and not seen observed until P5 in rat retina [25].

Functional significance of VGLUT3 expression in amacrine cells

Amacrine cells are the primary inhibitory interneurons that comprise the inner retina, and classically have been thought to release GABA or glycine [9,30,34,37]. It has been proposed by Johnson et al., [24] that VGLUT3 expressing amacrine cells release both inhibitory and excitatory neurotransmitters like starburst amacrine cells. Support for this hypothesis comes from studies in starburst amacrine cells showing that these cells contain acetylcholine in addition to GABA [6,49], and the released acetylcholine can function as an excitatory neurotransmitter on both nicotinic and muscarinic receptors in the inner retina [36]. In addition, amacrine cells in lower vertebrates have been found to contain high levels of glutamate [8, 40,52], with glutamate immunoreactivity present throughout all cell compartments, similar to the pattern of expression for VGLUT3 (see Fig. 1H and Fig. 2), suggesting the possibility that some amacrine cells store and release glutamate. However, unlike starburst amacrine cells these VGLUT3 immunoreactive amacrine cells also have been found to contain the inhibitory neurotransmitter glycine [21,24], and express the glycine transporter 1 [15,21]. Conceivably, these VGLUT3 expressing amacrine cells could function as both excitatory and inhibitory neurons, and have the potential to release both glycine and glutamate. In spite of this, a lack of vesicular inhibitory amino acid transporter expression in VGLUT3 expressing amacrine cells suggested that this cell might function solely in the extracellular uptake of glycine from the IPL [21,24]. Conversely, another study has shown that VGLUT3 amacrine cells are presynaptic to $\alpha 2$ glycine receptors [20], suggesting the possibility that glycine is released in addition to glutamate, perhaps through a non-vesicular mechanism. Therefore, in light of these equivocal observations and the development of VGLUT3 containing amacrine cells, which suggests that this amacrine cell synapse is unique from VGLUT1 or VGLUT2 containing synapses in the rat retina, further investigation is needed to determine whether glutamate or glycine is released from these amacrine cells and what impact this has on inner retinal processing.

EXPERIMENTAL PROCEDURE

Tissue Preparation

The retinas used for this study were obtained from male or female Sprague-Dawley rats (Charles River, Wilmington, MA). All experiments were performed in accordance with the guidelines for the welfare of experimental animals issued by the UCLA Animal Research Committee, and U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Rats were maintained on a 12 hr. light/ 12 hr. dark cycle and euthanized at the following time points: postnatal day 0 (P0) P1–P15, P20, P25, P30, P35, P40, P50 and P60. Animals younger than P8 were euthanized with cryoanesthesia and decapitated; animals older than P8 were euthanized with a lethal dose of Nembutal (80–90 mg/kg). For animals younger than P8, the eyes were enucleated and a cut was made into the cornea and the eyes were immersion-fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 15 minutes to 2 hours at room temperature. For animals older than P8, the eyes were cut along the ora serrata, the cornea, lens, and vitreous body were removed and the eyecups were immersion-fixed in 4% PFA. The eyes were then cryoprotected in 25% sucrose in 0.1 M PB. Prior to cutting the eyecup with a cryostat, the retina was washed with 0.1M PB, embedded in OCT compound (Sakura Finetek Inc., Torrance, CA) and rapidly frozen with dry ice or liquid nitrogen. Vertical sections of the retina were cut at 12–15 μ m mounted on gelatin-coated slides, air dried, and stored at -20°C .

Antibodies

Isoform specific antisera against VGLUT1 (Catalog No. AB5905), VGLUT2 (Catalog No. AB5907), and VGLUT3 (Catalog No. AB5421) (all raised in guinea pig, Chemicon, Temecula, CA) were used in this study. All of these antibodies have been previously characterized in rodent retinal tissue [VGLUT1 [25,41], VGLUT2 [25,41,50], and VGLUT3 [21,24]. All of these antibodies have been previously characterized in rodent retinal tissue [VGLUT1 [25, 41], VGLUT2 [25,41,50], and VGLUT3 [21,24]. The guinea pig antiserum against VGLUT1 (Cat. No. AB5905, Millipore, Temecula, CA) was raised against a synthetic peptide corresponding to C-terminal residues 542–560 of the rat sequence. The specificity has been tested by blocking with the specific peptide [50]. The guinea pig antiserum against VGLUT2 (Cat. No. AB5907, Millipore, Temecula, CA) was raised against a synthetic peptide corresponding to C-terminal region of the rat sequence. Preadsorption of the VGLUT2 antiserum with the immunogen peptide has been shown to eliminate all immunostaining in rodent retina [50]. The guinea pig antiserum against VGLUT3 (Cat. No. AB5421, Millipore, Temecula, CA) was raised against a synthetic peptide corresponding to of the rat sequence. The specificity of VGLUT3 has been tested by blocking with a GST-VGLUT3 fusion protein [24]. The optimal working dilutions were determined in adult retinal tissue, and the developmental studies were performed using half the optimal dilution for labeling of adult retina (VGLUT1 adult 1:80,000, developmental studies 1:40,000; VGLUT2 adult 1:8,000, developmental studies 1:4,000 VGLUT3 adult 1:6,000, developmental studies 1:3,000). This approach provides effective labeling of the antigen in developing tissue which typically contains lower expression levels of proteins in the synapse [7,28,29]. A rabbit polyclonal antibody raised against residues 659–672 from the COOH-terminal variable (V5) region of rat protein kinase C α was used to identify rod bipolar cells (1:200,000) [53] (Catalog No. P4334; Sigma Chemical St. Louis, MO). A rabbit polyclonal antibody raised against calcium binding protein 5 (CaBP5) (a generous gift from Dr. Françoise Haeseleer, University of Washington, Seattle, WA.) (1:2000) was used to identify bipolar cells terminating in the ON and OFF sublamina of the IPL [18]. A purified mouse monoclonal antibody against recombinant amino acid sequence 361–445 of the C-terminal binding protein 2 (CtBP2) (1:2000) (Catalog No. 612044; BD Transduction Laboratories, San Jose, CA), a RIBEYE homolog, that recognizes synaptic ribbons in mammalian retinas [39,48]. To check for antibody specificity, in the case

of single label experiments the primary antibody was omitted during the incubation step. In this case, only the nonspecific background staining is detected. In the case of double labeling experiments, omitting one of the two primary antibodies during the incubation should detect only the immunoreactivity for the remaining primary antibody and nonspecific background staining.

Immunohistochemistry

All tissue was labeled using the indirect fluorescence technique as described previously [23, 44]. Retinal sections were briefly dipped in 0.1 M PB before being warmed for 10 minutes at 37°C. Tissue was preincubated in a 0.1 M PB mixture containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X-100 for 1 hour. The sections were then incubated in primary antibodies overnight at 4°C, which were all diluted in 0.1 M PB (pH 7.4) containing 3% NGS, 1% BSA and 0.5% Triton X-100. The location of the primary antibody/antigen complex was detected using secondary antibodies conjugated to either Alexa 488, Alexa 568, Alexa 647 at dilution of 1:1000 (Molecular Probes, Eugene, OR). Following each antibody incubation, the retinal sections were washed for 10 minutes with 0.1M PB to remove any unbound primary or secondary antibody. For double or triple labeling experiments tissue sections were incubated in a mixture of primary antibodies followed by a mixture of secondary antibodies.

Confocal Microscopy

Images were acquired using a Zeiss 510 META Laser Scanning Microscope (Zeiss, Thornwood, NY) equipped with an argon laser for 488 nm excitation, and two HeNe lasers for 543nm and 633 nm excitation, respectively, and with a Plan Neofluar 63x 1.25 NA oil objective or a 40x Plan Neofluar 1.3 NA oil objective. During acquisition of signals from double-labeled or triple-labeled specimens, the scans were collected sequentially to prevent spectral bleed-through. Specific band pass filters were used to achieve proper separation of signals (for single labeling, 488/505LP, for double labeling 488/505–530, 543/560LP; for triple labeling, 488/505–530, 543/560–610, 633/650LP). Most images were acquired as 12 bit signals. To increase the signal-to-noise ratio, images were averaged online (e.g. n=4) and the scan speed and photo multiplier detector gain were decreased. Most confocal images were acquired at an approximate optical thickness of 0.5 μ m. For projections typically 10–20 optical sections were acquired with an average total thickness of 10 μ m and compressed for viewing. Some images have been deconvolved to remove out of focus fluorescence using an iterative deconvolution algorithm (Zeiss LSM software ver. 3.2). Digital confocal images were saved as Zeiss .LSM files and final publication quality images were exported in the .TIFF format. All images were processed and adjusted for brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA).

ACKNOWLEDGEMENTS

This work was supported by a Fight for Sight Student Fellowship (S.L.), a Fight for Sight Postdoctoral Fellowship (S.L.S., Jr.), and National Institutes of Health Grants: EY 04067 (N.C.B.), DK 41301, and a Senior Career Scientist Award from the Department of Veterans Affairs (N.C.B.).

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Figure 1. Immunoreactivity of VGLUT 1, 2, and 3 in adult rat retina

A–K: Digital images of vertical sections of the adult rat retina. **A–C:** VGLUT1 and PKC α immunolabeling. VGLUT1 expression is restricted to the OPL and IPL. PKC α labels rod bipolar cells, including the dendrites, soma, and terminal. **D–F:** VGLUT1 and CtBP2 (synaptic ribbon marker) immunolabeling in the OPL. VGLUT1 labels all photoreceptor terminals. **G:** VGLUT2 immunolabeling, shows strong signal in the somas of ganglion cells, and faint signal in putative Müller cell processes. **H–K:** Triple immunolabeling of VGLUT3-immunoreactive cells, with CtBP2, and CaBP5, a marker of ON and OFF bipolar cells. VGLUT3 showed a uniform morphology, with small, cell bodies, typically located near the inner edge of the INL, and slender processes descending to a broad plexus in the middle portion of the IPL. IS, inner

segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL ganglion cell layer. Scale bars = 10 microns.

Figure 2. Expression of VGLUT3 in the postnatal rat retina

A–D: VGLUT3 immunolabeling is not detected in the developing postnatal rat retina from P0 through P8. **E:** VGLUT3 immunolabeling first appears in a few amacrine cells at the inner edge of the INL at P10 with strong labeling in the somatic region (arrows) and few processes in the IPL. The signal in the somatic region (arrows) remains strong throughout development and in adult retina. **F:** By P15, the VGLUT3-immunoreactivity in the processes forms a continuous plexus within the middle portion of the IPL. **G, H:** The VGLUT3 labeling pattern becomes similar to the adult pattern by P20. NBL, neuroblastic layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars = 10 microns.

Figure 3. Expression of VGLUT1 in the postnatal rat retina. Vesicular glutamate transporter 1 (VGLUT1) is expressed in photoreceptor and bipolar cell terminals

A: VGLUT1 expression is absent during early postnatal development, from P0–P4. **B–C:** Weak VGLUT1 labeling is first detected in developing photoreceptor terminals at P5/6. **D:** Discretely labeled photoreceptor terminals are aligned in the developing OPL at P8. **C–D:** VGLUT1 labeling is faint at P6 and discrete labeling of the IPL is present at P8. **F:** VGLUT1 labeling is clearly labeling both the OFF and ON sublamina of the IPL by P10, inset shows a magnified image of IPL illustrating that VGLUT1 immunoreactivity is distributed to both the ON and OFF sublamina in the IPL. Dotted line divides the VGLUT1 expressing bipolar cell terminals in the OFF and ON sublamina. **G–I:** VGLUT1 labeling in both the OPL and IPL

increases in photoreceptor and bipolar cell terminals through remaining developmental stages. NBL, neuroblastic layer; OPL, outer plexiform layer; IPL, inner plexiform layer; Scale bars = 10 microns.

Figure 4. Expression of VGLUT2 in the postnatal rat retina. Increasing immunoreactivity in the ganglion cell layer is present from P0 throughout all developmental stages. Arrows indicate somatic immunolabeling in the GCL

A–B: VGLUT2 expression is faint at early postnatal development at birth from P0–P5. **C:** VGLUT2 labeling is prominent in the ganglion cell layer at P6. **D–E:** VGLUT2 labeling is observed in ganglion cells. **F:** Faint signals in the IPL and OPL appear around P10 and are likely indicative of Müller cell labeling. **G–I:** VGLUT2 labeling remains prominent in the GCL through adulthood. NBL, neuroblastic layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars = 10 microns.

Table

Antibody	Dilution	Host	Immunizing antigen	Remarks	Reference	Source
Vesicular Glutamate Transporter (VGLUT1)	1:40,000–80,000	Guinea pig (IgG)	Peptide sequence from rat VGLUT1	Labels terminals in photoreceptors and bipolar cells	Johnson et al., 2003; Sherry et al., 2003	Chemicon, Temecula, Ca. Catalog # AB5905
Vesicular Glutamate Transporter (VGLUT2)	1:4,000–8,000	Guinea pig (IgG)	Peptide from rat VGLUT2 sequence.	Labels ganglion cell somas, dendrites; faint Muller cell labeling; cone terminals; and horizontal cells	Johnson et al., 2003; Sherry et al., 2003; Wassle et al., 2006 Gong et al., 2006	Chemicon, Temecula, Ca. Catalog # AB5907
Vesicular Glutamate Transporter (VGLUT3)	1:3,000–6,000	Guinea pig (IgG)	Peptide from rat VGLUT3 sequence.	Labels amacrine cells	Haverkamp and Wässle, 2004; Johnson et al., 2004	Chemicon, Temecula, Ca. Catalog # AB5421
Protein Kinase C	1:200,000	Rabbit (IgG)	raised against residues 659–672 from the COOH-terminal variable (V5) region of rat protein kinase C α	Labels rod bipolar cells	Zhang and Yeh, 1991.	Sigma Chemical Corp., St Louis, Mo. (Catalog # P4334)
Calcium Binding Protein 5	1:2,000	Rabbit (IgG)	Recombinant mouse CaBP5	Labels rod bipolar cells, and some ON and OFF cone bipolar cells	Haeseleer et al., 2000	Dr. Francoise Haeseleer, University of Washington
C-terminal Binding Protein 2 (CTBP2)	1:2,000	Mouse (IgG)	recombinant protein consisting of amino acid sequence 361–445 at C-terminal binding protein 2	Labels the synaptic ribbon	Tom Dieck et al., 2005	BD Transduction Laboratories, San Jose, CA Catalog # 612044