

Expression of SNAP-25 during mammalian retinal development: thinking outside the synapse

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The SNARE complex is the core machinery required for vesicle fusion events. Numerous structural, functional, and genetic studies have led to a better understanding of mechanisms that regulate vesicle fusion events during neural development. Studies using the mammalian retina as a model system have increased our understanding of the dynamic patterns of expression of SNARE proteins. In particular, the SNARE complex protein SNAP-25 is expressed in a dynamic fashion during the development of cholinergic amacrine cells in a number of mammalian species. SNAP-25 is also likely to play a crucial role during the development of vertebrate photoreceptors. The integration of comparative studies examining SNARE proteins, such as SNAP-25, provides a powerful approach for the study of CNS development.

Key words: amacrine cells / *Monodelphis domestica* / photoreceptor / retina / SNARE complex

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SNAP-25: a SNARE complex protein

Vesicular fusion events are required for the release of neurotransmitter at the presynaptic terminal and for the incorporation of membrane material required for the growth of neuronal processes during brain development. Families of proteins have been identified that are essential for the fusion of vesicles with the plasma membrane, forming a core complex known as the SNARE (*N*-ethylmaleimide-sensitive factor attached

protein receptor) complex.¹ The SNARE hypothesis proposes that interaction of membranous vesicles with their target membrane is achieved via the binding of a VAMP (Vesicle Associated Membrane Protein) with SNAP-25 (Synaptosomal Associated Protein of 25 kDa) and Syntaxin, two proteins at the target membrane, thereby forming the SNARE complex.² This SNARE complex then serves as a binding site for other cytosolic and membrane-bound proteins that facilitate ATP-hydrolysis and vesicle fusion.³ Regulation and specificity of SNARE complex mediated vesicle fusion is likely due to their interaction with other proteins, differential expression of SNARE complex protein isoforms, their cell specific and subcellular localization, and their interaction with one another.^{4,5}

Regulation of SNARE complex protein expression appears to be critical during development of the nervous system.⁶ Numerous studies have defined a role for SNARE mediated vesicle fusion during neurite outgrowth.^{7–12} Further, the requirement of SNARE complex proteins for release of neurotransmitter¹³ strongly ties their expression to activity-dependent remodeling during neural development.

The mammalian retina as a model system: the developmental expression of SNAP-25

The retina is an ideal model system to examine the cellular and subcellular distribution of SNAP-25, as well as other proteins involved in vesicular release. All vertebrate retinas develop in a similar fashion and share the same basic laminar organization, consisting of three cellular layers (nuclear layers), separated by two synaptic layers (plexiform layers)¹⁴ (Figure 1). The outer nuclear layer (ONL) is composed of photoreceptors, while the cell bodies of horizontal, bipolar and amacrine cells, as well as the Müller glia, reside in the inner nuclear layer (INL). The retinal ganglion cell somata are located in the ganglion cell layer (GCL) and their axons form the

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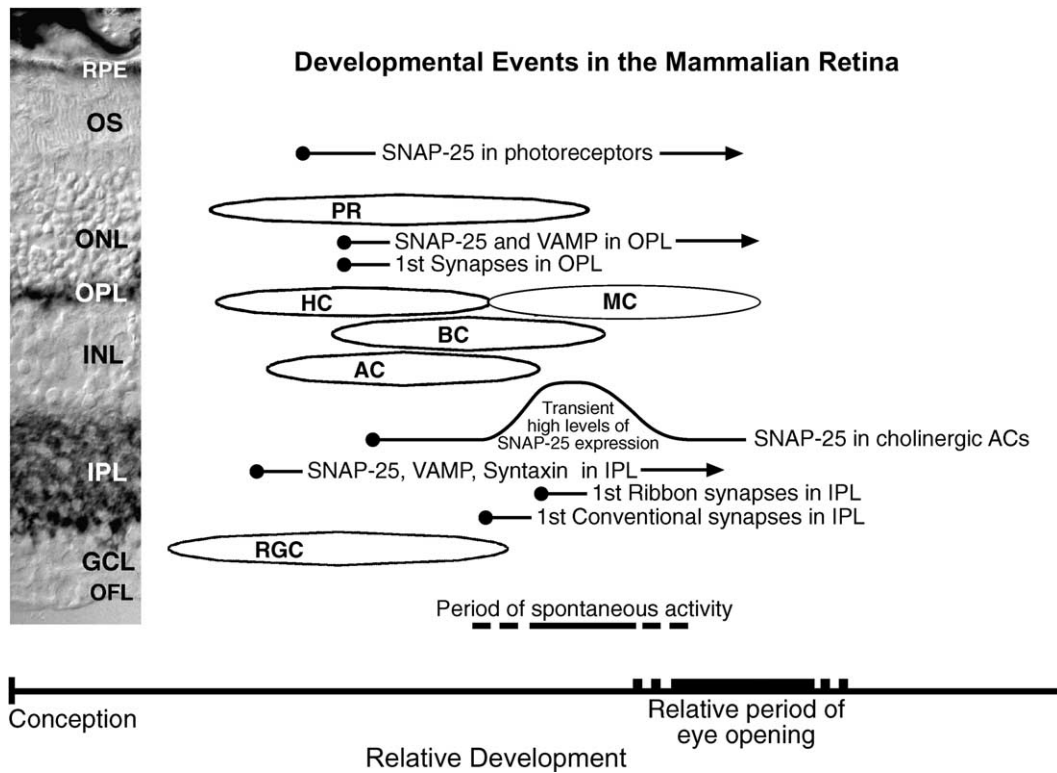


Figure 1. Summary of developmental events in the mammalian retina. This summary diagram illustrates the relative relationships of a number of developmental events in the retina and is based on studies from a variety of mammalian species including the Brazilian opossum,^{30,52} (Sakaguchi, manuscript in preparation), mouse (Wang *et al.*, manuscript in preparation), rat,⁵⁷ ferret,^{58,59} and cat⁶⁰. Additional information was obtained from Darlington and colleagues.⁶¹ The horizontal axis represents relative developmental time with respect to conception and the approximate time of eye opening. Black dots correspond to the approximate time when that particular event first appears. Elongated ovals represent the approximate timing of neurogenesis (for RGC, HC, PR, AC, BC) and gliogenesis (for MC). The image on the left is a section of a mature retina and the developmental events on the right are aligned with the appropriate retinal architecture. Abbreviations: RPE: retinal pigment epithelium; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; OFL: optic fiber layer; PR: photoreceptors; HC: horizontal cells; MC: Müller cells; BC: bipolar cells; AC: amacrine cells; RGC: retinal ganglion cells.

optic fiber layer (OFL), which also contains astrocytes. For each neuronal cell class, there exist multiple subtypes. Amacrine cells are the most diverse, with over 20 subtypes thus far identified based on their neurotransmitter¹⁵ or morphology.¹⁶ Each amacrine cell subtype has a distinctive arborization pattern of processes in the inner plexiform layer (IPL), for example, cholinergic, or starburst amacrine cells arborize in sublamina 2 and 4 of the IPL.¹⁷ Three morphologically different types of synapses are found within the plexiform layers, conventional,¹⁸ ribbon,^{19,20} and non-vesicular.²¹ SNAP-25 and VAMP-2 appear to function at all retinal synapses, while Syntaxin 1 is expressed in conventional synapses and Syntaxin 3 is

expressed in ribbon synapses.²² This synaptic diversity and characteristic development has made the mammalian retina an excellent model to study the expression and localization of SNARE complex proteins.^{22–28}

The study of SNARE complex protein expression in the retina provides a useful mean to compare retinal development between mammalian species. There are a number of species including a range of marsupials, mice, rats, rabbits, ferrets, cats, and primates that serve as model systems for visual system research. Studying patterns of SNARE complex protein expression provides a useful approach to evaluate *in vivo* and *in vitro* retinal development as well as the effects

of experimental manipulation.^{27,29} As the developmental functions for SNARE complex proteins continue to be elucidated, it is likely that many SNARE protein isoforms will exhibit transient patterns of expression, consistent with specific developmental roles. For example, we, as well as others, have characterized transient high levels of SNAP-25 expression in developing cholinergic amacrine cells in the Brazilian opossum,^{30,31} rat,²⁷ and mouse (Wang, Frishman and Sherry, personal communication) retinas. Depending on the duration of specific developmental events such as this, and the function of individual proteins, brief transient changes in levels of expression could be 'missed' in conventional model systems. Previously, we have taken advantage of the protracted period of postnatal retinal development in the Brazilian opossum, *Monodelphis domestica*³¹⁻³³ to characterize the changes in SNARE complex protein expression with greater temporal resolution. At birth, the *Monodelphis* retina is a relatively undifferentiated neuroepithelium with the earliest differentiating ganglion cells located in the dorso-central retina.³¹ Eye opening occurs around 35 days postnatal (35PN). Their immaturity at birth and their protracted period of postnatal development makes marsupials, like *Monodelphis*, excellent models for the study of visual system development.³⁴

Examples of cell-specific regulation of SNAP-25 in the developing mammalian retina

Transient high levels of SNAP-25 in cholinergic amacrine cells

Developing cholinergic amacrine cells, identified using an anti-choline-acetyl transferase (ChAT) antibody³⁵ transiently exhibit high levels of SNAP-25 immunoreactivity prior to eye opening^{27,30,31} (Wang, Frishman and Sherry, personal communication) (Figure 1). In the developing opossum retina, high levels of SNAP-25 expression parallel the onset of ChAT expression (Figure 2). Initially, all cholinergic amacrine cells are highly SNAP-25 immunoreactive, but as development proceeds, many cholinergic cells downregulate SNAP-25 expression, such that by eye opening, the majority have basal levels of SNAP-25 expression similar to surrounding cells (Figures 1 and 2). *In situ* hybridization studies suggest this decrease in SNAP-25 protein is regulated post-translationally.³⁶ The cholinergic amacrine cells appear to play a critical role in the generation and propagation of spontaneous waves of retinal activity that is essen-

tial for the normal development of the mammalian visual system.³⁷⁻⁴⁰ Transient high levels of SNAP-25 expression in these cells, therefore, temporally correlates with their period of participation in spontaneous retinal waves. This suggests that the regulation of SNARE protein expression and consequently vesicular release processes underlying neural activity may be instrumental for retinal development.

Prior to eye opening and normal visual stimulation, spontaneous waves of correlated activity are present in the developing mammalian retina.^{37,41} This spontaneous retinal activity appears to consist of two phases: an early cholinergic-dependent phase and a later glutamatergic-dependent phase.^{42,43} Blocking the cholinergic mediated spontaneous activity prevents the normal segregation of retinal ganglion cell axons into their appropriate eye-specific layers within the lateral geniculate nucleus of the thalamus.^{38,39} Retinal activity prior to visual stimulation is important not only for refinement of the visual projection but also for the establishment of appropriate circuitry within the retina itself. In the developing turtle retina, for example, blocking cholinergic-dependent retinal activity results in abnormally large ganglion cell receptive fields.⁴⁴ In the mouse retina, cholinergic-dependent activity plays a limited role in the segregation of retinal ganglion cell dendrites into ON and OFF strata of the IPL.³⁸ The involvement of cholinergic amacrine cells in spontaneous retinal activity may be facilitated by their relatively large, expansive arborizations that might allow communication of a single amacrine cell with many ganglion cells and other amacrine cells, via both chemical and electrical synapses.⁴⁵

The cholinergic-dependent spontaneous activity observed during the early development of the visual system may, in part, be regulated by differential expression of proteins that comprise the synaptic machinery necessary for regulated neurotransmitter release. SNAP-25 is crucial for regulated exocytosis of neurotransmitter.^{3,46} Although, SNAP-25 is clearly not essential for axonal innervation and initial synapse formation at neuromuscular junctions and in brain, earlier studies have implicated that this SNARE could play some role in the events that accompany the development of synaptic connectivity.^{8,47} The transient, high levels of SNAP-25 in cholinergic amacrine cells may represent a critical period for the selective enhancement of synaptic machinery in these neurons. Increased abundance of SNAP-25 may serve to facilitate the interaction of a large number of synaptic vesicles with the presynaptic terminal membrane, thereby increasing the likelihood of transmitter release.

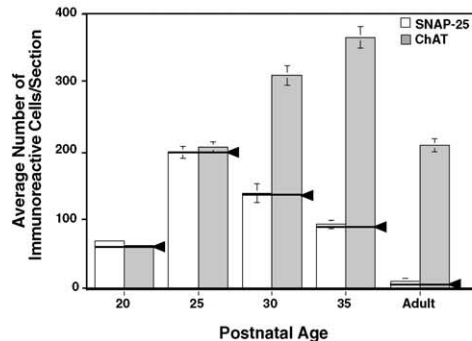
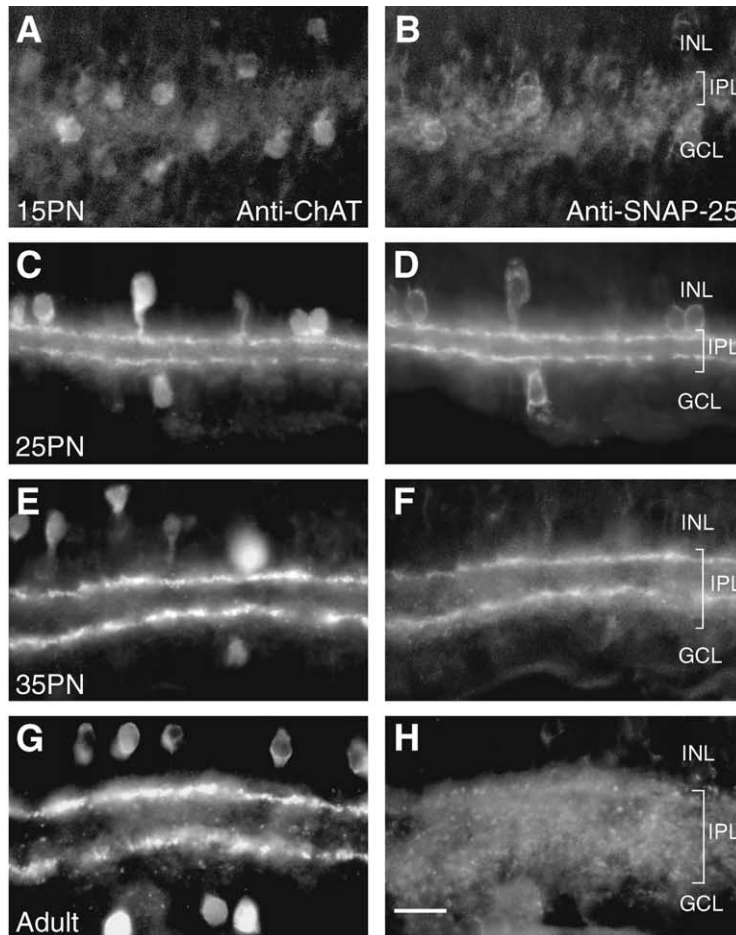


Figure 2. Transient high levels of SNAP-25 expression in developing cholinergic amacrine cells. Choline acetyltransferase-IR was first detected in the 15PN *Monodelphis* retina (A) shortly after high levels of SNAP-25-IR were first observed (B). In the 25PN retina, ChAT-IR (C) and SNAP-25-IR (D) were present in the same cells and processes in the IPL. At 35PN, just prior to eye opening, the distinctive pattern of ChAT-IR (E) in cell bodies and processes was evident, while high levels of SNAP-25-IR (F) in cell bodies were greatly diminished, though the bilaminar immunoreactivity in the IPL was still present. In the adult retina, ChAT-IR displayed its characteristic pattern (G), while SNAP-25-IR was now diffuse throughout the IPL and essentially absent from cell bodies (H). The graph represents the average number of ChAT-immunoreactive cell bodies and the average number of highly SNAP-25-immunoreactive cell bodies per section of retina from each age examined. The horizontal axis represents the ages examined and the vertical axis represents the average number of highly SNAP-25-immunoreactive or ChAT-immunoreactive cell bodies per section. The arrowheads pointing to dark, horizontal lines, represent the average number of double-labeled cells per section. Error bars represent SEM. Sample sizes for each age are as follows: 20PN ($n = 12$ sections from four eyes (12/4)); 25PN ($n = 17/6$); 30PN ($n = 15/5$); 35PN ($n = 9/3$); Adult ($n = 6/2$). Abbreviations: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer. Scale bar = 20 μ m.

Thus, the period of high SNAP-25 expression in cholinergic amacrine cells may represent a period of extensive synaptogenesis and/or synaptic plasticity for these cells.

High levels of SNAP-25 expression in photoreceptors

High levels of SNAP-25 expression have been observed in developing photoreceptors of the Brazilian opossum,^{31,33} rat,^{27,33} ferret,²⁴ and mouse (Wang, Frishman and Sherry, personal communication and manuscript in preparation) retinas (Figure 3). In the adult retina, high levels of SNAP-25 in photoreceptors are primarily localized to synaptic regions and inner segments.^{27,33} Photoreceptors are in a perpetual state of membrane shedding and renewal. This is the result of the continual addition of membranous discs to the base of the outer segment, which is necessary for maintenance of the outer segment, as outer segment membrane is lost when effete discs are regularly shed from the tip of the outer segment and phagocytized by the retinal pigment epithelium.^{48,49} In addition to its synaptic function

at the photoreceptor synapse,²² SNAP-25 in photoreceptors may play an integral role in the fusion of rhodopsin-containing vesicles with the inner segment membrane, which may in turn supply membrane for outer segment disc formation. This hypothesis is supported by reports that C-terminal mutations in rhodopsin resulting in its aberrant subcellular localization result in degeneration of photoreceptor outer segments.^{50,51}

Recent study of SNAP-25 null mutant mice has provided additional insight into the role for SNAP-25 in developing photoreceptors.⁵² Homozygous null ($-/-$) mice express no SNAP-25, while heterozygotes ($-/+$) express approximately 50% of SNAP-25 as compared to wild-type (wt) animals.⁴⁶ Retinae of heterozygotes were examined by immunostaining for the protein recoverin⁵³ which can be used as an early marker for photoreceptors in the developing retina. Mice, expressing 50% of normal SNAP-25 levels ($-/+$), have an average of 33% more recoverin-immunoreactive cells than wt littermates at embryonic ages (Figure 4). This suggests that mice with a lower level of SNAP-25 expression exhibit an

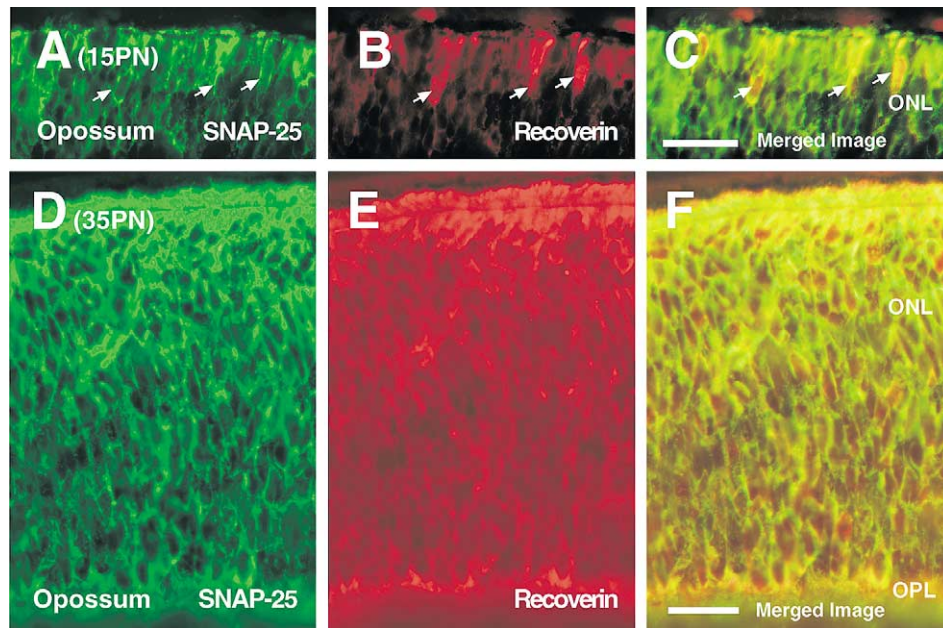


Figure 3. High levels of SNAP-25 are expressed in developing photoreceptors. During early retinal development, in the 15PN *Monodelphis* retina, high levels of SNAP-25 were present in most cells in the outer retina, while recoverin-IR was observed in only a few cells in the outer retina (A and B). Merging the two images demonstrates that these recoverin-IR cells were also SNAP-25-immunoreactive (C, arrows). Just prior to eye opening, at 35PN in the *Monodelphis* retina, the vast majority of ONL cells were immunoreactive for SNAP-25 and recoverin (D and E). Merging images D and E demonstrates co-localization of SNAP-25 and recoverin-IRs (F). Abbreviations: PN: days postnatal; OPL: outer plexiform layer; ONL: outer nuclear layer. Scale bars: A–C = 10 μ m, D–E = 20 μ m.

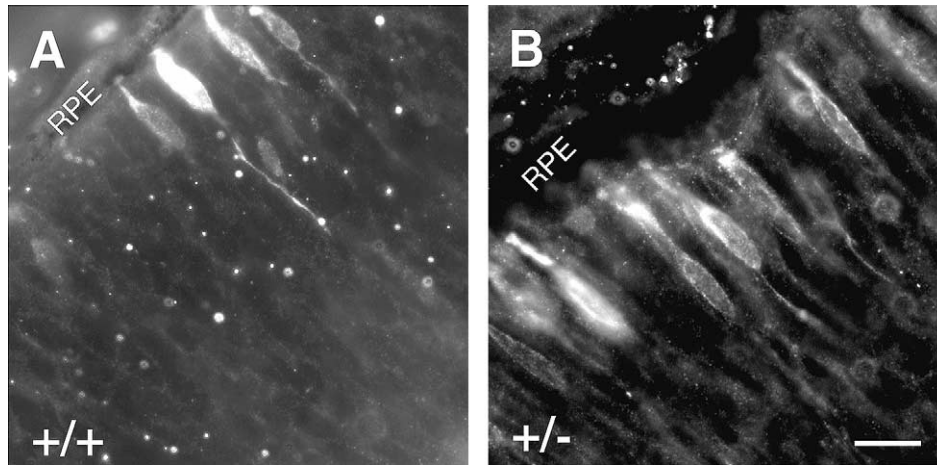


Figure 4. Normal levels of SNAP-25 expression in the developing retina are crucial for appropriate photoreceptor differentiation. Transgenic mice heterozygous for the SNAP-25 null mutation display 50% of normal levels of SNAP-25 expression. Recoverin-immunoreactivity identifies differentiating photoreceptors in the E16.5 wt retina (A). In a heterozygous littermate, the density of recoverin-immunoreactive cells is much higher (B). Within a given litter, the heterozygotes have an average of 33% more recoverin-immunoreactive cells compared to their wild type littermates. Abbreviations: (+/+): wild-type; (-/+): heterozygote; RPE: retinal pigment epithelium. Scale bar = 20 μm .

increase in photoreceptor differentiation during this early period of retinal development (manuscript in preparation). In addition, preliminary functional studies of juvenile heterozygote retinas suggest an increase in cone function. Taken together, these data suggest that SNAP-25 regulation in developing photoreceptors is important for appropriate photoreceptor differentiation. Furthermore, decreased SNAP-25 expression in the photoreceptors of the heterozygotes may somehow favor cone development. This effect may be due to a decrease in vesicular release of a factor that normally suppresses photoreceptor differentiation during embryonic retinal development.^{54,55} Cells of the embryonic retina also express cell surface receptors for factors that inhibit differentiation of photoreceptors. For example, the Delta–Notch signaling pathway acts to inhibit differentiation of retinal progenitor cells.⁵⁶ These receptors may be exposed to the cell surface via fusion of a receptor-containing vesicle with the plasma membrane. A decrease in SNAP-25 expression might then decrease the fusion of these receptor-containing vesicles, and effectively reduce the signaling via such an inhibitory pathway. While the precise effect of a decrease in SNAP-25 expression in the developing retina is still under investigation, it seems clear that high levels of SNAP-25 in the developing retina are critical for the appropriate differentiation of photoreceptors.

Conclusions

SNARE complex proteins play critical roles during regulated vesicular release of neurotransmitter. Furthermore, they have important roles during neurite outgrowth and synaptogenesis. The spatiotemporal localization of SNARE complex proteins provides new criteria for the comparison of retinal development between mammalian species and to evaluate retinal development *in vitro* or within other experimental paradigms including transgenic animals.

This review has discussed the differential expression of SNAP-25 in two specific cell types within the mammalian retina. During development, spontaneous waves of activity drives the segregation of retinal ganglion cell axons into eye specific lamina in the lateral geniculate nucleus. The transient high level of SNAP-25 expression in cholinergic amacrine cells correlates with their participation in spontaneous retinal activity. While a direct functional correlation has not yet been established, the developing retina may provide an exceptional opportunity to study the functional effect of an increase in expression of vesicular release machinery. High levels of SNAP-25 in photoreceptors may serve two distinct functions. High levels of SNAP-25 in the photoreceptor inner segment suggest SNAP-25 may play a crucial role in fusion of rhodopsin-containing vesicles with the inner

segment membrane. High levels of SNAP-25 in photoreceptors during development, however, appear to function at some point during cell fate determination and therefore may be important for photoreceptor differentiation. It is clear that the characterization of SNAP-25 expression in the developing retina may afford additional opportunity for the study of SNAP-25 function 'outside the synapse'. For example, one particularly intriguing question is whether the activation of the protein machinery mediating these developmental events is, like synchronous neurotransmission required for synaptic communication, dependent on membrane depolarization and calcium influx. Ongoing and future studies examining the dynamic patterns of SNAP-25, as well as other SNARE complex proteins in the retina and their relationship to activity-dependent synaptic plasticity and cellular differentiation, will likely provide invaluable information relevant to mammalian CNS development.

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