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CONVERSATIONS WITH RAY GUILLERY ON ALBINISM: LINKING SIAMESE CAT VISUAL PATHWAY CONNECTIVITY TO MOUSE RETINAL DEVELOPMENT

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Abstract

In albinism of all species, perturbed melanin biosynthesis in the eye leads to foveal hypoplasia, retinal ganglion cell misrouting, and, consequently, altered binocular vision. Here, written before he died, Ray Guillery chronicles his discovery of the aberrant circuitry from eye to brain in the Siamese cat. Ray's characterization of visual pathway anomalies in this temperature sensitive mutation of tyrosinase and thus melanin synthesis in domestic cats opened the exploration of albinism and simultaneously, a genetic approach to the organization of neural circuitry. I follow this account with a remembrance of Ray's influence on my work. Beginning with my postdoc research with Ray on the cat visual pathways, through my own work on the mechanisms of retinal axon guidance in the developing mouse, Ray and I had a continuous and rich dialogue about the albino visual pathway. I will present the questions Ray posed and clues we have to date on the still-elusive link between eye pigment and the proper balance of ipsilateral and contralateral retinal ganglion cell projections to the brain.

Graphical Abstract

This conversation with Ray Guillery on albinism includes his description of the discovery of the aberrant connections between eye, thalamus and cortex in the Siamese cat, which harbors a temperature-sensitive mutation of tyrosinase and thus, only the most distal structures are pigmented. Carol Mason chronicles her time as a postdoc with Ray, and her studies on the developmental steps in the albino mouse leading to a similar misrouting of retinal axons to the dorsal lateral geniculate nucleus.

Keywords

retinal ganglion cell; albino; tyrosinase; melanin; retinal pigment epithelium (RPE); axon guidance; transcription factor; visual cortex; lateral geniculate nucleus

INTRODUCTION

This article is an unusual contribution to the personal reflections and scientific reports in this special issue in memory of Ray Guillery, for he wrote a large part of it. In 2015, Ray was contacted to write a review on albinism and asked me to join him. His contribution to that unfinished article (Part I) tells the story of his discovery of how defects in pigment in the eye lead to aberrations in circuitry from the retina to the thalamus and the cortex, with the Siamese cat as a model. In his own first person voice, Ray describes his discoveries in his inimitable clear and precise style. Ray delights in the serendipity of his journey, and his essay exposes his musings, critical analysis, and humor better than any of us could have done. In Part II, I describe my experiences with Ray and his effect on my work. Part III recounts my lab's journey to probe how retinal ganglion cell (RGC) axons decussate at the optic chiasm in pigmented mice. I then relate these analyses to our studies of the albino mouse retina and visual pathway: In the albino mouse retina, an altered pace of RGC neurogenesis is linked to altered RGC specification. RPE cell integrity is also perturbed and may dysregulate communication of still unknown signals between the RPE and neural retina that direct neurogenesis and specify ipsi- or contralateral RGCs. These defects set the stage

for RGC misrouting at the optic chiasm and abnormal visual mapping in the thalamus and cortex of albinos that Ray detailed so well.

Chris Walsh (this issue) states Ray's core questions on albinism: "...the *Siamese cat* presents two fundamental and fascinating problems in developmental biology. One relates to how retinal ganglion cells are instructed to project to one side or another of the brain, and the role of pigment—which is only obviously present in the pigmented retina and not in the neural retina—in this process"; these questions are addressed in Part III. "And the second developmental problem, much more profound, is how map reorganization prior to experience takes place." The former question has been my interest, inspired by Ray. The latter question was Ray's focus, described in Part I. The articles in this issue by Murray Sherman, Anthony LaMantia, Zoltan Molnár, Jeremy Taylor, and Chris Walsh also touch on Ray's pioneering approach to linking genes, circuits and behavior (Sherman, 2017; LaMantia, 2018; Molnár, 2018; Taylor, 2018; Walsh, 2018). And for additional reviews on the albino, see Jon Kaas' excellent narrative of Ray's discovery (Kaas, 2005), and comparisons of the albino rodent and human visual systems (Jeffery, 2001) and of the albino, achiasmatic, and pigmented visual systems (Prieur & Rebsam, 2017). Below is an account of Ray's discovery, in his own words.

I. TRACKING THE ACTIONS OF A SINGLE GENE FROM THE EYE THROUGH THE BRAIN (Ray's title for our article)

1) Finding an abnormal visual pathway and identifying the relevant gene

When in 1969, working in the Anatomy Department of the University of Wisconsin at Madison, I (RG) found a strange and unexpected abnormality in the visual pathways of a cat, I did not know that I was dealing with the action of a well-known and already well-studied gene, the albino gene, nor that one of geneticists who had contributed significantly to the study of this gene more than half a century earlier (Wright, 1915; Crow, 1990) was in Madison and that we would be discussing this abnormality some months later over lunch. It was probably a conversation with the eminent geneticist, Jim Crow (later Dean) at the Beer and Beefsteak club (still men only when I first got to Madison), at which I found out that Sewell Wright actually had retired from the University of Chicago to Madison, and was getting to be close to 100... he was 99 when he died!

The abnormality in the Siamese cat was striking. Whereas in a normal cat, about 60% of the fibers that link the retina to the brain cross the midline on their way to the cerebral cortex, 40% stay uncrossed (Figure 1A). They pass through a well-studied relay, the lateral geniculate nucleus (LGN) which has several separate, well defined layers in cats, and primates: one set of layers in the right LGN receives a topographically organized input from the left nasal retina and another set of layers receives from the right temporal retina (see Figure 1A, where only one layer of each set is shown). The right LGN sends fibers from both sets of layers to the right occipital cortex so that this receives an orderly, topographically organized input from the left visual hemifield of both eyes. The two sets of geniculate inputs, one from each eye, are matched in the cortex so that any one small patch of cortex receives matching inputs from the visual scene.

I had cut the retinofugal axons from one eye and was studying the distribution of their degenerating terminals in the LGN, trying to understand the arrangement of the layers and their innervation in the cat. In one unusual, apparently abnormal cat, the degenerating axons from one eye went not only to the geniculate layers that normally receive from the contralateral eye, but also to a part of the layers that normally receive from the ipsilateral eye. And those two segments receiving crossed inputs were fused near the midline where the vertical meridian of the visual field is represented (Figure 1B at the asterisk). These contralaterally innervated layers were distinctly separated from the small part of the layers that still received input from the ipsilateral eye.

At first, I was puzzled and tried to work out whether some artifact of the preparation had created this seemingly false image. I worked with Barbara Hoege, the technician who had prepared the sections and everything was in order, and there were no folds, air bubbles, staining failures, or other conceivable artefacts. I was lucky: I had recorded that this was a Siamese cat. I remembered that Siamese cats were commonly cross-eyed or strabismic. This feature of Siamese cats is more common in Europe, where I grew up, than in the US, where “abnormalities” in pets are less sympathetically viewed. Siamese cats carry a temperature dependent variant of the albino gene that produces pigment only over the coldest parts of the body: the tips of the ears and the feet as well as the scrotum. I learnt that Siamese cats that spend time outdoors in the Moscow winter become dark all over the body. Siamese cats resemble the Himalayan rabbit in their pigment distribution, as do mutant forms of the guinea pig studied by Sewall Wright (Wright, 1915). He had demonstrated the range of albino alleles in guinea pigs and their pigment patterns. A further point I learnt as I started to take an interest in this genetic abnormality, was that the albino gene is a recessive gene that codes for the enzyme tyrosinase (Durham, 1904; Wright, 1915; Witkop *et al.*, 1970; Oetting, 2000), which is an enzyme crucial in pigment formation in the skin and the eyes including the iris and the pigment epithelium of the eye, although, oddly, not in the substantia nigra, which produces melanin using a different enzyme, tyrosine hydroxylase. Perhaps most important was the knowledge that human albinos, like Siamese cats, are commonly cross-eyed and have weaknesses of vision that in the past often put them in schools for the visually handicapped.

A study of a second Siamese cat confirmed that, indeed, the abnormal fiber crossing and the abnormal layers of the lateral geniculate nucleus closely matched those of the first cat. We had been taught the rule (Beadle & Tatum, 1941) “one gene, one enzyme,” and it looked as though here was a single gene, responsible for an identified enzyme, that produced abnormalities of pigment production as well as abnormalities of the visual pathways that at the time had no known link with pigment production at all. I knew that a single gene could have multiple actions because I had been taught genetics by Hans Grüneberg in London. He had shown that “multiple effects of a single gene, ‘pleiotropy’ as it was then called, were really multiple secondary effects of a single primary action” (Lewis & Hunt, 1984) and it looked as though this single enzyme could have multiple, quite unexpected, actions in the brain of all places, where a different enzyme (tyrosine hydroxylase) went on quietly making the pigment of the substantia nigra that had no relationship to the visual pathways.

There were a number of intriguing questions. How could the action of the one gene have such unexpected different effects in the brain? Specifically, how did pigment production relate to control of the retinofugal crossing? How did the ocular origin of retinogeniculate axons relate to the formation of geniculate layers? And perhaps, most significantly from a functional point of view and for the program of our research group then in Madison, how did these cats see the world? This abnormal crossing pattern was a marked change from the normal partial crossing, which had been recognized already by both Newton and Descartes as essential for producing a single cerebral representation from the binocular input coming through lenses that invert the image of the world on the retina (see Figure A). The altered Siamese pattern could not possibly (it seemed) be producing a normal single cerebral representation of the visual scene. How were these cats seeing the world? Where to start?

2) Thinking about gene action

The action of genes at the time seemed of particular interest. Studies of genes in *Caenorhabditis* (Brenner, 1973) and in *Drosophila* (Benzer, 1971) were making great strides in showing that specific genes produced particular neural and behavioral outcomes, based on the “belief that the structure and function of the nervous system, and hence the behavior of an animal, is specified by its genes.” This quotation is from Gunther Stent (Stent, 1981), who described this as the “ideological approach” of genetics, i.e. using the information about genes to understand developmental neurobiology and behavior. He contrasted this to the “inductive approach”, which used the genetically produced outcomes as tools, comparable to experimental lesions or alterations “to help expose the ground rules of the developmental process.” Stent rightly saw that “the case of the Siamese cat reveals the conceptual poverty of the notion that genes specify the neural circuits that underlie behavior.” He wrote: “*All that the gene mutated in the Siamese cat can usefully be said to “specify” is the amino acid sequence of an enzyme that takes part in melanin synthesis, presumably in retinal epithelial cells..... despite these specific cerebral aberrations there is, in this paradigmatic case, no trace of genetic specification of neural circuitry.*”

There were many issues that needed to be addressed instrumentally in terms of Stent’s usage. Perhaps the most immediate question concerned the role of the pigment in mis-routing the retinogeniculate axons at the optic chiasm. However, the most puzzling for us at the time was how a Siamese cat sees the world.

3) How does a Siamese cat see?

In order to look at the way in which a Siamese cat sees, I (RG) joined Jon Kaas, who, in a different part of the same building, was busily mapping cortical visual fields at the time. He and I decided to record from the primary visual cortex of Siamese cats. This was at the time that several groups had already produced extensive studies of the visual cortex of normal cats (Hubel & Wiesel, 1971; Woolsey, 1971; Clarke & Whitteridge, 1976) and all we had to do was to find out what happened to the out of register and inverted maps shown in the Figure for the LGN when they were passed to the visual cortex. What we found seemed pretty straight-forward. The normally connected geniculocortical projection had essentially taken over the cortex (Figure 1C). Only very few of the abnormally innervated geniculate cells—that is those that received a reversed order of the visual inputs (as indicated the

reversed arrow in the lower right of Figure 1B) were functionally linked to the visual cortex. And the small lateral normal segment was poorly represented as well. The Siamese cats had a normal field of view, but it was predominantly monocular. The large abnormally crossed retinogeniculate component had essentially no functional representation in the cortex. It looked as though there had been an executive decision that the abnormal input makes no sense, so best to ignore it.

While we were doing these studies, we heard occasional reports of Hubel and Wiesel giving talks about cortical maps in Siamese cats that were quite different. At first, it was hard for us to interpret the verbal reports, but when Hubel and Wiesel published their results, they were clear and strikingly different from ours. Where, in the medial parts of the LGN, at the asterisk, the normal and abnormal contralaterally innervated geniculate layers are fused along the representation of the vertical meridian of the visual field, it was as though this midline fusion acted like a hinge in the middle of a continuous representation of the visual field (Figure 1D). This included, as normal, the contralateral part of the visual field, and then continued into the (abnormal) ipsilateral part of the visual field. This continuous representation had been passed to the cortex (the hinge had been opened) and in the cortex Hubel and Wiesel reported that the abnormal geniculate segment (receiving from the ipsilateral visual field) was now represented in the most medial part of the primary visual cortex, where the vertical meridian is normally represented. The representation of vertical meridian and the normal representation of the contralateral hemifield had been shifted laterally in the cortex and the two parts now formed a continuous map of the greater part of the visual field. The normal, lateral part of the LGN (that is the most peripheral part of the visual field) was represented laterally as in a normal cat. These Siamese cats had corrected the abnormal visual field map in the LGN by changing the geniculocortical pathways. There were significant parts of the visual cortex that received an abnormal monocular input, either from the normal or from the abnormal sector of the LGN. These cats (like our cats) had no true binocular vision comparable to the normal cat (Figure 1A). The strange “correction” of the inverted arrow seemed improbable, but Jon and I took Hubel and Wiesel’s results very seriously even though they were at odds with what we saw.

We argued that for the pathways described in normal cats and in our Siamese cats, the geniculocortical pathways in both should show essentially the same pattern of retrograde geniculate cell degeneration after a lesion in the binocular part of the cortex, provided that the abnormal (reversed) projections still sent their axons to the cortex but failed to drive the cortical cells. The zone of geniculate cell loss should include all of the layers in the affected segment of the LGN, its borders passing straight through all the layers. If, indeed, some Siamese cats showed the geniculocortical pattern shown in Figure 1D, then a cortical lesion should produce a monocular zone of cell degeneration in just one (monocular) part of just one set of geniculate layers. Any lesion of segments 9–12 in Figure 1D would produce degeneration in just one set of geniculate layers.

We made a number of lesions in the visual cortex of Siamese cats (Kaas & Guillery, 1973). Most showed a normal pattern of retrograde geniculate degeneration, confirming our results and also showing that the geniculate axons from the abnormal geniculate segments reached the cortex, but had failed to drive the cortical cells. One of the Siamese cats with a cortical

lesion showed an essentially monocular segment of geniculate degeneration such as would be expected from Hubel and Wiesel's account, clearly distinct from the findings in normal cats and in our other Siamese cats.

We concluded that for most of our Siamese cats, the normal geniculocortical projection was preserved and the cats suppressed the abnormal, conflicting visual inputs to the cortex from the abnormal LGN. We called this the "Midwestern pattern." In the one unusual cat in our colony and in all of the cats studied by Hubel and Wiesel, the geniculocortical projection was "corrected" so that a new continuous representation of the visual field was generated. We called this the Boston pattern. Carla Shatz (Shatz, 1977) confirmed the distinction between the Boston and the Midwestern cats, reporting from the Harvard lab that one of 12 Siamese cats showed the Midwestern pattern. She also demonstrated that the callosal connections of the visual cortex, which in normal cats link the representations of the vertical meridian of the visual field at the border between the first and the second cortical visual areas, in the Boston Siamese cats is shifted away from the border, where vertical meridian is represented as shown in Figure 1D by the asterisk.

The Boston pattern relied on the functional continuity of the representation of the visual input within the geniculate layers (see Figure 1B) dominating and influencing the development of the geniculocortical pathway. The Midwestern pattern relied on maintaining the normal topography of the geniculocortical pathway. We don't know how the dominance of one or the other of these developmental processes is established during development, but it may well be that they both develop at about the same time. In a normal cat, they would reinforce each other, since both produce the same result. Which came first and dominated the development of the geniculocortical pathway would make little difference in a normal cat. In the Siamese cats, the developmental sequence may well be crucial for determining the dominance.

With Vivien Casagrande (Guillery & Casagrande, 1977), we showed that a normally reared Midwestern Siamese cat does not respond to visual stimuli falling on the temporal retina, confirming the results of the cortical recordings. In these Siamese cats, a monocular lid suture results in normal visual responses to stimuli falling on the temporal retina of the open eye, demonstrating that the loss of vision in the temporal retina is due to competition between the non-matching visual inputs from the two eyes to the cortex. Packwood and Gordon (Packwood & Gordon, 1975) showed that normal cats have stereoscopic vision, but that Siamese do not. We then showed a comparable chiasmatic abnormality in an albino monkey linked to abnormal laminar fusion (Guillery *et al.*, 1984). Creel (Creel *et al.*, 1974) and Apkarian (Apkarian *et al.*, 1984) demonstrated that albino humans showed abnormalities of the visual evoked potentials that matched the expected chiasmatic abnormalities. All albino mammals that have been studied, including rats (Lund, 1965), mice (LaVail *et al.*, 1978), guinea pigs (Giolli & Creel, 1973), rabbits and ferrets (Sanderson, 1975), mink (Sanderson *et al.*, 1974), a tiger (Guillery & Kaas, 1973) and a wallaby (Guillery *et al.*, 1999) show the abnormal chiasmatic crossing described here. There is also some evidence that the auditory pathways may be involved as well (Conlee *et al.*, 1984; Baker & Guillery, 1989).

4) Forming abnormal fusions and separations of geniculate layers

Figure 1 shows that the abnormal fusions and separations of geniculate layers accord with the novel continuities and discontinuities of retinal representations established by the abnormal retinogeniculate pathways. Wong (Wong, 1999) had demonstrated waves of activity that pass across the retina during early development at stages when the geniculate layers are being developed. She has pointed out that these waves involve the retinal ganglion cells and amacrine cells and could thus play a role in grouping geniculate cells in accordance with the retinal origins of their inputs. The abnormal laminar patterns are in accord with this suggestion, although the actual cellular controls that can produce cellular continuities and discontinuities as the geniculate layers develop are currently not known.

Ray's account of his discoveries on the albino visual circuitry ends here. Throughout, Ray considered both the cellular and genetic constraints as well as the way this biological framework is further impacted by activity and experience. As he wrote in the above section, he believed that retinal waves and neural activity contribute to establishing geniculate layers and, in the albino, adjusting connectivity in the cortex, but he did not test this supposition in his lifetime. Current investigations of the role of retinal waves and neural activity in the visual pathways, now done largely on the mouse, have established that activity cooperates with molecular cues to establish the topographic organization of visual maps in a retinotopic and eye-specific manner (Seabrook *et al.*, 2017; Thompson *et al.*, 2017; Lorusso *et al.*, 2018). The intricacies of the mechanisms underlying such activity-dependent modification of circuits are still unclear. How the visual pathway connectivity "adjusts" in the Siamese visual cortex from the misrouted, fused and abnormally segregated retinogeniculate inputs as Ray outlined has not been addressed. Analysis of the ipsilateral retino-geniculo-cortical pathway and participation of the tenurin family of molecules (Leamey & Sawatari, 2014) could be carried out to further illuminate the role of neural activity in such consolidation of circuitry, at the very least, in the albino mouse.

Ray's enormous contribution was to introduce the albino mutation as a way to understand the organization of pathways for binocular vision/depth perception beyond image-forming vision. And, although he did not study the development of these pathways, he predicted the steps and underlying mechanisms for how binocular vision is established at the pathway and system level beyond topography, as discussed in Part III below.

II. TIME WITH RAY – LAUNCHING MY JOURNEY

My (CM) connection with Ray was from the start independent of the albino question. With Ray, I focused on deciphering neuronal connections from electron micrographic data. Ray wrote me when I was in England, his adopted homeland, to inquire whether I would like to work with him and Tony Stretton at the University of Wisconsin to relate the form of neurons to their synaptic relationships in *C. elegans*. I had a background in dye-labeling invertebrate neurons and linking morphology and connectivity (Mason, 1973), and extended these to analysis of connectivity of neurosecretory cells in the rodent hypothalamus. I jumped at the chance to work with Ray in a second postdoctoral position and to use the newly installed 3-story tall High Voltage Electron Microscope (EM). I also yearned to return

to the States to experience the new field of neuroscience, just as the Society for Neuroscience was getting started.

When I joined Ray's lab in 1976, I found a place where I could luxuriate in describing the microscopic anatomy of individual neurons. The High Voltage EM studies did not take off, but John Robson, another postdoc in Ray's lab, Grayson Scott, Ray's long-time EM staff scientist, and I adapted a method for linking the light microscopic structure of axonal branches and boutons with their synaptic connections. We did this by bulk filling axons with horseradish peroxidase (HRP) to yield beautifully detailed morphology of retinogeniculate axons compared with the capricious Golgi impregnation, and correlated bouton form with their synaptic relationships with correlated LM-EM analysis (Mason & Robson, 1979; Robson & Mason, 1979) (Figure 2). John and I went on to use this approach to study axon sprouting in the denervated lateral geniculate nucleus (LGN) (Robson *et al.*, 1978; Bickford, 2018).

Ray then gently steered me toward a more independent path, into the terrain where I am firmly planted ever since: unveiling the basics of axonal pathway development. Studying growth cones in the visual system of the developing cat (Mason, 1982b; Mason, 1982a) poised me for examining growth cones in the visual pathway of the more accessible mouse, specifically, at the optic chiasm. Ray's intellectual guidance impacted my studies on the developing visual system and cerebellum and deeply influenced my thinking then and now. First, he cultivated my proclivity for observation and observing "natural history," my interest since my undergraduate work in marine biology, on how to take notes while examining every section before photographing them, gleaned and "reconstructing" data from the sections in one's mind, making predictions about the organization of neurons, and pushing farther than others had gone before. I learned to explore and to persist in unraveling puzzles, such as whether retinotopy is maintained in the optic tract before retinal axons arrive at their targets. I tackled this question with Ray and another postdoc Fernando Torrealba (Torrealba *et al.*, 1982) after making lesions in the cat retina and silver staining to track degenerating axons. We gained more traction on the topic recently; with fluorescent dye- and genetic labeling we investigated eye-specific organization of the mouse optic tract (Lee *et al.*, 2018; Sitko *et al.*, 2018).

Ray could also be removed and a bit cynical, yet he guided us with tough love, no matter one's gender nor standing in the lab. He was critical and pushed us intellectually, often with a twinkle in his eye. He did not direct, but let us grow and explore on our own. Although rarely effusive, Ray could be quietly supportive and even proud. When he asked me to show the Chinese scroll-like drawing I made of a single axon in the kitten dLGN to the visiting David Hubel, his aloofness dissolved. Ray himself was remarkably self-effacing, eschewing self-promotion. He scorned the then new medium of "pop" reviews, which he thought supported one's ego (what would he think of social media!). I recall Ray referring to one of the first review journals "Trends in Neuroscience" (TINS) as "Rumors in Neuroscience" as I was preparing a piece for TINS in 1984. However, he must have come around in that view, since he himself later wrote several pieces for TINS, among them (Mitrofanis & Guillery, 1993).

Four years later, I left Ray's group, by then in Chicago, to start my own lab at NYU. I wanted to continue delving into development. Ray convinced me to explore the cerebellum, arguing it would be much easier to unravel because of the regularity of cell layers and known synaptic connections. Armed with his paternal encouragement and with my propensity to mine single cell morphology and synaptic relationships, I worked on the cerebellum for the next decade. I probed the early synaptic relationships of mossy and climbing fibers (Mason & Gregory, 1984). With Mary Beth Hatten, we examined neuron-glial interactions, modeling them *in vitro* (Mason & Gregory, 1984; Gregory *et al.*, 1988). Our collaboration culminated in an analysis of granule cell-Purkinje development by mixing and matching purified cells, to test their mutual contribution to Purkinje cell development (Baptista *et al.*, 1994) – all without any knowledge of the molecules that mediated the interactions.

In the 80's and 90's, I returned to the visual system, and studied glial development in the accessible retina to optic nerve and chiasm setting. To distinguish glia from axons in the optic nerve in the electron microscope, Paola Bovolenta and I returned to HRP labeling of retinal ganglion cells (RGCs) in the eye. This topic was also taken up by Ray with beautiful electron microscopy, without markers, to show the changes in form and arrangement of immature glia as retinal fibers entered the chiasm area (Colello & Guillery, 1998). Then, although not looking for them, we observed growth cones in the nerve and into the chiasm, with forms never before revealed. This led us as many other labs at the time studying invertebrate and vertebrate nervous systems (Tosney & Landmesser, 1985; Caudy & Bentley, 1986; Williams *et al.*, 1991) to demonstrate what Cajal had reported in the spinal cord: that growth cone form reflects their behavior and is associated with sites where that behavior changes, for instance, during retinal axon decussation at the chiasm and entry to targets (Bovolenta & Mason, 1987). By encouraging the cellular neuroanatomist in me, Ray thus launched my exploration of visual system development, leading us to probe how retinal axon decussation is directed at the optic chiasm.

III. DEVELOPMENT OF THE IPSILATERAL AND CONTRALATERAL PROJECTION IN THE PIGMENTED AND ALBINO MOUSE VISUAL SYSTEM

Ray tackled the question of abnormalities in the visual system circuitry of the Siamese cat as a model for albinism and brilliantly showed how connections are modified to suppress the mis-information. He pondered developmental aspects of retinal axon decussation, but did not further pursue the molecular mechanisms underlying pigmented and albino pathway development. Instead, in the 2000's, he dove into his work on the thalamus in collaboration with Murray Sherman (Sherman, 2017). Nonetheless, at the time he worked on the circuitry problem in the Siamese cat, he raised crucial, still valid questions pertaining to visual pathway development: How does the ocular origin of retinogeniculate axons relate to the formation of geniculate layers? And, how does pigment production relate to control of retinofugal crossing? He left us with these questions which I will address in this section.

a. DEVELOPMENT OF RETINAL AXON DECUSSATION AT THE OPTIC CHIASM: PIGMENTED MICE

Proper binocular vision depends on the routing at the optic chiasm of the correct proportion of RGC axons that project to the same (ipsilateral) and opposite (contralateral) side of the brain. Ray's prescience was our guide to probing the mechanisms of visual pathway development, aptly stated in a review he wrote with Jeremy Taylor and me (Guillery *et al.*, 1995): "*As the axons pass from the eye to the chiasm they tend to lose their retinotopic order, to gain a chronotopic order, and then, in the region of the chiasm, to regain some aspects of the retinotopic order before reaching their terminal sites. Molecular or cellular cues that allow the several distinct organizational steps to occur must be expected in the retina, on the axons themselves, and also along the pathway of the axons, prechiasmatically and at the chiasm. Some of these cues will be associated with local nerve cells, some with specialized glial elements and some with the retinofugal axons themselves.*"

These predictions, combined with my earlier studies in Ray's lab on the cat and kitten visual pathways helped focus our work on growth cones along the optic pathway onto the establishment of the binocular pathway, specifically at the optic chiasm. What followed was a 25 year-long journey seeking to understand how RGCs are instructed to project to one side of the brain or the other, in "normal" pigmented and albino mice. In our review (Guillery *et al.*, 1995), Ray went on to say that "*several candidate molecules have been identified in the retina and along the path of the axons, but to date no clearly defined role in the specific events of the pathway determination have been identified.*" While true in 1995, we now know a great deal about the cellular and molecular factors contributing to the path RGC growth cones take to lay down the binocular circuit (Petros *et al.*, 2008; Erskine *et al.*, 2017).

1) RGC growth cone forms reveal how decussation is accomplished

In the early 90's, we teamed up with Pierre Godement, who had serendipitously discovered that the carbocyanine dye DiI, even when applied to fixed tissue, beautifully fills every nook and cranny of neurons and their processes. Although the labeled neurons had to be observed with fluorescence microscopy, the ability to dissect the precise area of dye application with a stereo microscope made it possible to reveal the endings of growing axons or growth cones in developing tissue as well as their cell origins because DiI also travels retrogradely. In the late 80's, Pierre visited us to show very large and complex growth cones he saw *only* after labeling the ventrotemporal (VT) retina, source of the permanent ipsilateral cells (Godement *et al.*, 1990; Godement *et al.*, 1994). These images were even more astonishing than those revealed by our earlier HRP labeling. And drawing the growth cones, as was customary from Golgi and HRP or other labeled material, was incomparable for divulging the behavior in these static preparations. Sadly, this custom of camera lucida drawing of cell morphology has died out with the advent of fluorescent microscopy.

2) Chronicles of the behavior of RGC axons in the optic chiasm

In living preparations of eyes and optic stalks attached to the ventral diencephalon, we were able to visualize uncrossed RGC axons after DiI deposition in the VT retina and crossed axons by labeling all other poles of the retina. Ipsi- and contralateral RGC axon behavior was thereby brought to life with video time-lapse microscopy. Both ipsi- and contralateral

axons slow as they approach the midline, and both populations advance and retract over a period of an hour or two. The most complex growth cones positioned proximal to the optic chiasm midline, expand and seem pressed up an “unseen glass partition,” as described in a News & Views article about our live imaging after presenting our work at the Society for Neuroscience in the early 90’s. After an hour or two, the ipsilateral growth cones regroup and turn away from the midline, and the contralateral axons cross the midline and take off rapidly (Godement *et al.*, 1990; Godement *et al.*, 1994; Mason & Wang, 1997). The waiting with small advances and retractions of both crossed and uncrossed fibers near the midline reminded us of Ramon y Cajal’s description of growth cones that he observed in fixed preparations, as “soft battering rams”.

3)

The live imaging was the foundation for identifying the ***cellular cues for RGC divergence at the optic chiasm***, a “decision region” or intermediate target, a locus in neural pathways often at the midline, much studied in the field at the time. With immunohistochemistry of molecules first observed in the spinal cord floor plate (and studied by my Columbia colleague, Jane Dodd), we identified a radial glial palisade draping the chiasm midline and a plate of early-born neurons intersecting the glial cells (Marcus *et al.*, 1995; Marcus & Mason, 1995). This cellular ensemble expresses many epitopes similar to the floor plate and cortical subplate, all three sites where transformation, or changes in direction or tempo (e.g., pausing), occurs. Interestingly, these sites are comprised of cells that are transient (Mason & Sretavan, 1997). Moreover, there are domains within the ventral diencephalon, in which the chiasm is formed, expressing transcription factors that may control the guidance molecules expressed by them (Marcus *et al.*, 1999) and if missing, will lead to a complete lack of an optic chiasm (Seth *et al.*, 2006).

4) **Guidance receptors on RGCs that interact with factors in the optic chiasm for RGC divergence**

The early 2000’s was the era of molecular discovery. Inspired by studies in invertebrates and vertebrate model systems on axon guidance, especially at the midline of the neuraxis, we identified molecules expressed by the cells of the optic chiasm to which RGCs had receptors and were candidates for repulsion from or attraction to and across the chiasm midline (Plump *et al.*, 2002). The creation of *in vitro* assays of retina and chiasm cells facilitated testing candidate guidance factors expressed *in vivo*. Clues to the system of receptors and ligands that function in retinal axon divergence came from Christine Holt’s lab. Holt discovered that in *Xenopus*, when tadpoles metamorphose into a frog, a small ipsilateral projection forms and an ephrinB is expressed at the chiasm midline and an EphB receptor is expressed in ventrotemporal RGCs (Nakagawa *et al.*, 2000). The cues important for RGC decussation were then confirmed to be analogous in the mouse: EphB1 receptors are localized exclusively and fleetingly on ipsilateral RGCs in the VT retina, and ephrinB2, the ligand for EphB1, on midline glia in the chiasm (Williams *et al.*, 2003). The EphB1-ephrinB2 interaction results in the inhibition of ipsilateral growth cones from VT RGCs from extending farther at the chiasm midline (Figure 3).

Cues for the guidance of the contralateral projection include NrCAM and Semaphorin6D on midline glia and chiasm neurons, and NrCAM and Plexin-A1, both receptors to Semaphorin6D, on RGCs (Kuwajima *et al.*, 2012). The Kuwajima study was one of the first in the field to implicate a consortium of receptors and ligands at a given locale, and, in fact, this consortium expressed on both growing axons and the terrain through which they grow, that acts synergistically for axon growth and guidance. Recently, other canonical and new guidance systems functioning in parallel have been reported for optic chiasm midline decussation (Erskine *et al.*, 2017; Peng *et al.*, 2018) (Figure 3).

After mingling with each other in the optic chiasm, crossed and uncrossed fibers immediately segregate in the proximal optic tract (Sitko *et al.*, 2018). The molecular underpinnings of this segregation are beginning to emerge, and include dystroglycan (Clements & Wright, 2018), matrix molecules (Reese *et al.*, 1994; Reese *et al.*, 1997; Hornberg *et al.*, 2016), glial cells (Reese *et al.*, 1994; Lee *et al.*, 2018), and elements important for local translation of guidance proteins (Hornberg *et al.*, 2016), but a strict expression pattern associated with the ipsi- vs contralateral RGC populations has not been established. Our *in vitro* analyses have shown that mouse ipsilateral RGCs have a greater propensity to fasciculate with their own “kind” compared with contralateral RGCs (Sitko *et al.*, 2018). Single cell genomic analyses should reveal candidates for such behavior leading to eye-specific axon segregation in the optic tract.

5)

In the early 2000's, ***transcription factors expressed by specific neuronal populations with specific projection patterns*** were discovered (Polleux *et al.*, 2007). One proposed function of such transcription factors, still holding today, is to direct the expression of the guidance factors - receptors on the growing axon and ligands in cells in their paths, on which similar or complimentary transcription factors are expressed. Ray asked the question during our interchanges as to whether “*one needs two sets of factors, and whether one can argue that the transcription factors guide axons.*” The ipsilateral RGCs in VT retina express different receptors to axon guidance ligands, compared to the contralateral RGCs mainly positioned outside of the VT crescent. Recall that Ray also proposed that “*differences must exist between ganglion cells at different retinal loci to govern the different axon behaviours that have been described*” (Guillery *et al.*, 1995). While there is no evidence that the transcription factors themselves guide axons, the tenet that the two populations have different molecular programs also applies to the specific transcription factors expressed in the retina: Ipsi- and contralateral RGCs have distinct transcription factors guiding both their differentiation and guidance factor expression.

In 2003, we identified Zic2 as a transcription factor regulating the ipsilateral RGC program of development. Zic2 is expressed in RGCs destined to project ipsilaterally, limited to the VT retina, and it regulates expression of EphB1 and SERT, the latter used during activity-dependent targeting and refinement in the thalamus (Herrera *et al.*, 2003; Garcia-Frigola & Herrera, 2010). Ray wrote a positive and informative News and Views about our discovery of Zic2 (Guillery, 2003). Evidence for these factors relating to RGC chiasmatic choice was found for mice, ferrets, frogs, the latter when they develop an ipsilateral projection at

metamorphosis (Herrera *et al.*, 2003), and in humans (Lambot *et al.*, 2005), in all cases, correlated with the proportion of RGCs that project ipsilaterally (Herrera *et al.*, 2003). Thus *Zic2* is a signature gene for ipsilateral RGCs (Figure 3) and has been a major entre into probing the albino phenotype (see III.b.).

A search for a corresponding transcriptional program for contralateral RGCs, considered the evolutionarily default pathway, culminated in our identification of the SoxC family (Sox4, Sox11 and Sox12), important for the expression of Nr-CAM and PlexinA1 and thus contralateral RGCs but not ipsilateral differentiation (Kuwayama *et al.*, 2017). The SoxCs supplanted *Islet2* (Pak *et al.*, 2004) and *Brn3a* (Muzyka *et al.*, 2018) as regulators of the contralateral RGC program, as *Islet2* and *Brn3a* are expressed only in subsets of contralateral RGCs (Figure 3). We also performed gene profiling of ipsi- and contralateral RGCs and found, in addition to transcription factors, genes for growth factors and their receptors expressed in a complimentary fashion in ipsi- and contralateral RGCs such as *Igf1* (contralateral) and *Igfbp5* (ipsilateral) RGCs (Wang *et al.*, 2016). Similarly, *Shh* is expressed in contralateral RGCs and the *Shh* receptor component BOC in ipsilateral RGCs (Sanchez-Arrones *et al.*, 2013). These findings on transcription factors associated with distinct RGC classes are likely an indicator of a far more multi-factorial transcriptional “state” that undoubtedly includes the same sort of “consortium” of transcription factors referred to above for guidance molecules. Advances in single cell profiling promise to reveal more genes specific to RGCs with regard to their laterality of projection, and when and how these genes function in ipsi- contra cell fate, fasciculation and guidance, and targeting (Rheume *et al.*, 2018). These studies stimulated interrogation of how these developmental steps go wrong in the developing albino visual system (see III.b.).

6) The time and place of ipsi- and contralateral RGC neurogenesis is critical to their specification

In many neural systems, cell type specification is linked to the time of birth (Tripodi *et al.*, 2011; Osterhout *et al.*, 2014). Different cell subtypes express specific sets of transcription factors, and each functional or projection type subclass has a specific period of production. We know the developmental history of the ipsi- and contralateral RGCs (Dräger, 1985; Colello & Guillery, 1990). Ipsilateral RGCs in pigmented rodents are born in a defined period; the VT retina subsequently reverts to producing contralateral cells (Marcucci *et al.*, 2018). A precise birth order proceeds for functional RGC classes, the X, Y, W cells in higher vertebrates (Walsh *et al.*, 1983).

Our gene profiling of ipsi- and contralateral RGCs revealed that a cell cycle regulator, Cyclin D2, is enriched in the ciliary margin zone (CMZ) at the retinal periphery (Wang *et al.*, 2016). Cyclin D2 is thought to be important for maintaining cells in an intermediate state (Glickstein *et al.*, 2009; Tsunekawa *et al.*, 2014) and is expressed in subpopulations of cells giving rise to different interneuron populations in the cortex (Petros *et al.*, 2015). Similarly, Cyclin D2 is critical for the output of RGCs from the ventral CMZ, particularly ipsilateral RGCs (Marcucci *et al.*, 2016).

Former postdoc, Eloisa Herrera and her lab performed live imaging of the CMZ in embryonic retinal slices. After migrating radially from the apical aspect of the retina

adjacent to the RPE to the basal CMZ, some cells translocate laterally from the CMZ to populate the neural retina (Marcucci *et al.*, 2016). These findings raise the question of whether the CMZ, long known to be a site where retinal cells are continually produced in amphibia and fish as their retina enlarges, may provide RGCs in higher vertebrates, and all or a subpopulation of ipsilateral RGCs. We also determined, as reported previously (Dräger, 1985), that the ipsi- and contralateral RGC populations are born at distinct times, and that the VT retina lags behind the rest of the retina in neurogenetic output. Moreover, in the absence of Cyclin D2, cell cycle exit in CMZ is impaired (Marcucci *et al.*, 2018). Thus, the CMZ and the cell cycle regulator Cyclin D2 comprise a system which, if perturbed, could result in a discrepancy in the size of RGC populations that project ipsi- or contralaterally. This hypothesis indeed forecasts the situation in the developing albino mouse retina.

b. DEVELOPMENT OF RETINAL AXON DECUSSATION AT THE OPTIC CHIASM: ALBINO MICE

Throughout his work on the Siamese cat, Ray realized the importance of the albino mutation for understanding the organization of pathways for binocular vision. Particularly relevant to our work are the reduction of the ipsilateral RGC axonal projection, and a concomitant increase in the contralateral projection, hallmarks of all forms of albinism. Although Ray did not decipher the link between defects in melanogenesis in the RPE and the reduced ipsilateral RGC projection, he correctly hypothesized that the altered circuits were the result of developmental events and experience-dependent rearrangements of ipsi- and contralateral inputs to the thalamus and cortex. Here, I present our current understanding of several of these events in the albino mouse visual system, in particular, how RGC neurogenesis and cell fate specification in the pigmented mouse go awry. I will also relate how the lack of melanin in the RPE may affect both early RPE cell integrity and in turn, neural retinal development, resulting in perturbed central connectivity of retinal axons.

Ray and I discussed new findings on pigmented and albino visual system development by email and in person, on a last visit in Oxford in 2015 as we walked through the “Narnia” woods near Ray’s home, near where C.S. Lewis had once lived and where Lewis wrote his *Chronicles* books. Ray posed the questions listed below on the albino visual pathway development and I provide some answers to these questions, with the hope of producing clues on the enigma that is albinism.

1) Is retinogeniculate connectivity altered in the albino mouse?

When we began to work on the albino in the mid-90’s, we did not intentionally seek to compare the misrouting and altered circuitry phenotype of the Siamese cat with that of the albino mouse visual system. In the late 2000’s, newer tracing techniques facilitated a more precise comparison of the retinogeniculate projection in albino and pigmented mice, by injecting each eye with differently colored fluorescent tracers such as cholera toxin, at postnatal ages. This approach revealed an aberrant patch of fibers in the albino dorsal LGN adjacent to, but segregated from, the terminal field of RGC axons that normally extend from the contralateral VT retina late in embryonic development (Rebsam *et al.*, 2009; Rebsam *et al.*, 2012). While the configuration of terminal zones in the dLGN in pigmented and albino mice is different from that of the Siamese cat, the principal features of the phenotype are

similar: in the Siamese cat, more contralateral fibers extend to the dLGN, and layer A enlarges and fuses into layer A1. A smaller terminal zone of the normal ipsilateral fibers forms in layer A1, and the misrouted fibers from the contralateral eye in layer A1 are segregated from the ipsilateral fiber zone (see Ray's description in Part I., Figure 1; graphical abstract). In the albino mouse, the segregated patch of terminals adjacent to the dorsal tip of the dLGN is composed of terminals of a larger than normal cadre of RGCs residing in VT retina that project contralaterally, similar to the "hinge" that is in layer A1 in the Siamese cat LGN (Figure 1). Strikingly, the segregation of the aberrant patch in the mouse dLGN is activity-dependent (Rebsam *et al.*, 2012), an aspect that Ray predicted for the Siamese cat but could not test. Of further interest is that in the mouse, the increased RGCs that abnormally project contralaterally derive from the VT retina. These RGCs must retain recognition molecules that enable targeting to the dorsal tip of the dLGN, the correct retinotopic site for contralateral RGCs from the VT retina. The fact that the fibers aberrantly crossing the midline in the Siamese cat also derive from temporal retina is indicated by the fusion of the medial region of layer A to layer A1, normal targets of the temporal retina (Figure 1). Thus in both species, more RGCs, from temporal retina, are fated to be contralateral rather than ipsilateral.

2) Which genes reflect a reduction of ipsilateral RGCs?

When we first discovered that *Zic2* is a gene expressed exclusively in ipsilateral RGCs in VT retina, we immediately examined the albino retina. In another "aha" moment, we found fewer *Zic2*-expressing RGCs in the VT retina of albino mice, exactly matching the reduction in the ipsilateral component (Herrera *et al.*, 2003) (Figure 4). The *Zic2* expressing RGCs in both pigmented and albino retina are located in the same ventral temporal domain, but in the albino are less frequent. Further, there are more RGCs expressing *Islet2*, a marker for contralateral RGCs (Bhansali *et al.*, 2014). These findings illustrate that upon a reduction in the specification of ipsilateral RGCs, contralateral RGCs are concomitantly increased presumably from a fate switch, and alterations in retinogeniculate connectivity ensue (see III.b.1).

3) Is timing of neurogenesis also altered in the albino retina and does it affect fate specification?

Previously, it was thought that abnormalities in the projection from eye to brain in the albino could be due to altered neurogenesis (Ilia & Jeffery, 1999; Kralj-Hans *et al.*, 2006) and retinal maturation (Webster & Rowe, 1991). To better analyze perturbations in neurogenesis and/or differentiation, we used birthdating combined with cell-specific markers, first, *Islet1/2* for RGCs in albino and pigmented mice (Rachel *et al.*, 2002a). Relative to pigmented mice, more ganglion cells are born in the early stages of neurogenesis in the albino retina, and the cellular organization of the albino retina is perturbed as early as E12. Interestingly, the temporal aspect of the retina is expanded in albino relative to the position of the optic nerve head. These reports suggested spatiotemporal defects in neuronal production in the albino retina.

We returned to such an analysis with newer birthdating methods and *Zic2* as a marker for ipsilateral cells, and investigated the spatiotemporal dynamics of RGC production in relation

to diminished ipsilateral RGC number in the albino retina (Bhansali *et al.*, 2014). We found that the peak of RGC genesis and differentiation in the albino VT retina is delayed by approximately a day compared with pigmented retina. This delay could perturb expression of genes that specify cell fate (Figure 4). We therefore inferred that the aberrant cluster of axon terminals in the albino dLGN reflects an increase of RGCs in the VT retina specified to a contralateral fate, reflected in the increased number of RGCs expressing Islet2, potentially because these RGCs were born too late to express Zic2, the nodal ipsilateral gene.

What controls the timing of neurogenesis of ipsi- and contralateral populations in the pigmented retina and, is this control dysregulated in the albino? Cyclin D2 in the CMZ is a candidate for such a regulator as described above (III.a.). In the albino, indeed, fewer cells in the CMZ express Cyclin D2 (Marcucci *et al.*, 2016) (Figure 4), likely leading to fewer ipsilateral RGCs produced and a concomitant reduced ipsilateral projection, and resembling the phenotype of pigmented retina when Cyclin D2 is missing (Marcucci *et al.*, 2016). These results suggest that disruption of pigmentation in the RPE is associated with altered tempo of ipsi- vs contralateral RGC neurogenesis, altered specification and fate, and consequently, the reduced and misrouted ipsilateral projection to targets observed in albinism. We are currently investigating in the albino whether the reduction in Cyclin D2 expression produces defects in cell cycle exit (Marcucci *et al.*, 2018), whether there is a delay in Cyclin D2 expression reflecting the delay in neurogenetic output, and whether RGC production from the CMZ (Belanger *et al.*, 2017) is altered.

4) Does melanin, or its perturbation, link to visual pathway abnormalities in the albino, and if so, what is the mechanism?

This question is the holy grail in understanding the mechanisms that lead to an imbalance of ipsilaterally- and contralaterally-projecting RGCs in albinism. Melanin forms just as the eye cup invaginates, at E11 in mouse, and is localized in melanosomes in the retinal pigment epithelium (RPE). Early work had correlated melanin levels with the size of the abnormal crossed projection (Sanderson, 1975; LaVail *et al.*, 1978; Creel *et al.*, 1982). That is, fully albino animals show a larger abnormal crossed component than animals having milder pigment abnormalities, such as in Siamese cats and Himalayan rabbits, arguing for a direct relationship of melanin to laterality of RGC axon projection.

Melanin is also expressed elsewhere in the visual pathway, leading Ray and his lab, along with the groups of Jerry Silver and Carla Shatz, to ask whether pigment in the optic stalk and/or in the optic chiasm was relevant to guidance to the pathway of the retinofugal axons (Silver & Sapiro, 1981; Strongin & Guillery, 1981; Webster *et al.*, 1988; Colello & Jeffery, 1991). This early set of analyses ultimately resulted in placing the onus on pigment in the eye itself, fortified by experiments in our lab in which retinal explants were cocultured on wild type or albino chiasm cells; the albino retina showed greater defects in neurite growth, irrespective of the genotype of the chiasm cells (Marcus *et al.*, 1996). In parallel, Hector Chan and Ray demonstrated that the optic tract and chiasm were organized similarly in albino and pigmented mice (Chan *et al.*, 1993), and based on the smaller VT RGC component in the albino retina, they too inferred that the albino gene has its primary action in the retina, a conclusion that holds to this day. In further support of this conclusion, in the

mink, coat color is irrelevant, only eye color matters (Sanderson *et al.*, 1974), but in humans the relationship is less clear (Reissmann & Ludwig, 2013).

Further experiments focused on the requirement for melanin. Attempts were made to rescue the abnormality by genetically replacing tyrosinase, important for melanin synthesis, in albino mice and demonstrating a more balanced ipsi-contra retinogeniculate projection (Cronin *et al.*, 2003). In fact, only very low levels of tyrosinase are needed for melanization of the RPE and a normal ipsilateral projection (Rachel *et al.*, 2002b). However, abnormality does not depend specifically on the lack of the enzyme, tyrosinase, since both tyrosinase negative (e.g., Oculocutaneous albinism, OCA) and tyrosinase positive (e.g., Ocular albinism type I, OA1) (Palmisano *et al.*, 2008; Young *et al.*, 2011) forms of albinos show similar visual abnormalities, including a reduced uncrossed projection (Preising *et al.*, 2011) and seem not to depend on the lack of the pigment itself or disrupted melanogenesis, but in the case of OA1, the turnover of melanosomes. To this end, Ray recounted “*Carl Witkop (Witkop et al., 1970), a dermatologist and geneticist, was treating an African American couple who were both albinos and who had a normally pigmented child. People, including their doctor, told the father that this must be an out of wedlock baby. The father challenged Carl, by saying that you doctors may not know all the answers; for all you know my wife and I are different sorts of albinos. Carl took this seriously and tested a few hairs from each parent for tyrosinase. One was positive the other was negative.*”

Ray wondered, “*might we then conclude that melanin itself is irrelevant, but rather, that the process of producing melanin is crucial?*” Further studies focused not on melanin but on L-Dopa, which is produced in the pathway from tyrosine to melanin by the action of tyrosinase on tyrosine to produce l-dopaquinone and dopamine. Replacement of tyrosine hydroxylase in the RPE of albino mice also rescued the retinogeniculate projection (Lavado *et al.*, 2006). That L-Dopa is involved in defects associated with albinism was further supported by biochemical analyses showing that the embryonic albino RPE is nearly devoid of L-Dopa (Roffler-Tarlov *et al.*, 2013). Administering L-Dopa via a pregnant mouse mothers’ drinking water can lead to uptake in the albino eye (Roffler-Tarlov *et al.*, 2013). Rescue of the reduced number of Zic2⁺ cells occurred after L-Dopa delivery to the mother, but in some cases, hyperproliferation of retinal cells was observed (Mason, Bhansali, Roffler-Tarlov *et al.*, unpublished), likely due to L-Dopa’s role in cell proliferation (Ilia & Jeffery, 2000; Reis *et al.*, 2007). However, attempts to correct visual defects in humans with Levodopa were ineffective (Summers *et al.*, 2014), potentially because the treatments were done well after RGC neurogenesis. Other experiments have argued that L-Dopa is a ligand for the orphan GPCR on the melanosome surface encoded by the OA gene (Lopez *et al.*, 2008; McKay, 2019). These latter studies point to factors relevant to maintenance and health of mature RPE, and have not elucidated the relationship between factors in the RPE during embryogenesis and the specification of ipsi vs contra RGCs.

Other recent work argued not only against melanin, but even its synthetic pathways as primary factors in producing the albino mis-routing phenotype. Mutations in *SLC38A8*, encoding a glutamine transporter, in humans cause the foveal and decussation abnormalities phenotype in the retina and visual pathway mimicking those in albinism, but *without* pigment loss or perturbation (Poulter *et al.*, 2013). Additional signaling pathways in the

RPE, unassociated with the melanin pathway directly, such as *au18* and *au13* in zebrafish, encode a protein required for the maturation of melanosomes and cause albinism (Hanovice *et al.*, 2015). Further, deletion of the U3 ubiquitin ligase, *Mindbomb*, in the RPE impacts retinal neurogenesis by perturbing Notch signaling, but it is not known whether *Mindbomb* deletions would lead to an albino phenotype (Ha *et al.*, 2017). These pathways are well worth examining for a role in affecting neurogenesis, cell specification and RGC misrouting that are seen in the albino phenotype.

A crucial question from all of the work to date is whether melanin or molecules in the melanin synthesis pathways, act directly on RGC neurogenesis, or indirectly by primarily affecting RPE cell integrity, junctions and maintenance. In the latter case, additional signals from the RPE would direct retinal cell development. There could be a convergence of such pathways to processes downstream that lead to an imbalance of ipsi-contra RGC axon trajectory. This convergent step could involve regulation of neurogenesis and gene expression that together confer laterality of axon navigation and projection, as discussed in III.a.1)-3), or processes such as patterning of the early retina, CMZ and RPE, as discussed in III.b.5) below.

5) Does aberrant melanogenesis affect optic vesicle development and subsequently, the development of and interactions between the RPE and neural retina?

During early eye development, after invagination of the eye anlage, the optic vesicle differentiates into two closely apposed layers, the RPE and the neural retina (Raymond & Jackson, 1995; Martinez-Morales *et al.*, 2004; Bharti *et al.*, 2006; Fuhrmann *et al.*, 2014). In the postnatal vertebrate eye, an intact and functional RPE is crucial for photoreceptor outer segment phagocytosis, photoreceptor survival, and retinoid metabolism. Genetic ablation of the RPE during embryogenesis results in disrupted morphogenesis and lamination of the neural retina (Raymond & Jackson, 1995), suggesting that the RPE is also required for proper neural retinal development. However, how the RPE and developing neural retinal layers interact to influence retinal development and RGC neurogenesis, and how the two layers might fail to interact in the albino retina, are poorly understood.

In the mouse, the RPE acquires pigment beginning at embryonic day (E) 11.5. Ipsilaterally- and contralaterally-projecting RGCs are born thereafter. Irrespective of whether an RPE-derived factor is allied with melanin synthesis or with other processes important for RPE health and maintenance, how would these factors affect neurogenesis and cell fate in the neural retina? Intercellular junctions are positioned between RPE cells and between RPE and neural retinal precursors as they divide to produce the several layers of the adult retina. These junctions could be important conduits for signaling molecules that regulate RPE integrity and the development of the neural retina, respectively (Jeffery, 1998; Cook & Becker, 2009), and that allow the passage of small molecules from cell to cell, such as ATP, which can control retinal proliferation as studied in wild type rodents (Pearson *et al.*, 2005). The localization and phosphorylation state of the gap-junction forming protein Cx43 are defective in the embryonic albino RPE (Iwai-Takekoshi *et al.*, 2016) (Figure 4). Gap junction phosphorylation state is critical for cell-cell communication and controls proliferation in cardiac myocytes (Solan & Lampe, 2014), and proper channel gating is

critical for transfer of beneficial factors. Thus, the perturbations in Cx43 phosphorylation seen in the albino could affect RPE integrity itself and damage the underlying neural retina through a by-stander effect, leading to the disrupted neurogenesis, cell identity, and misrouting of RGCs through the optic chiasm to thalamic targets comprising the albino phenotype. Interestingly aberrant gap junction expression in the albino can be alleviated with L-Dopa (Tibber *et al.*, 2007), which can travel through gap junctions due to its relatively small size, compared with the larger melanin molecule.

Morphogenetic factors such as FGFs and Wnts regulate the development and patterning of the CMZ, RPE and neural retina. Wnt signaling (Fujimura *et al.*, 2009; Westenskow *et al.*, 2009; Alldredge & Fuhrmann, 2016), the Yap/Taz pathway (Miesfeld *et al.*, 2015), and Pax6 (Cohen-Tayar *et al.*, 2018) are important for RPE cell fate, and FGF is crucial for neural retina fate (Guillemot & Cepko, 1992; Pittack *et al.*, 1997; Picker & Brand, 2005). The interaction between FGF and Wnt signaling has recently been chronicled (Balasubramanian, 2018). Whether mutations of these genes affect RGC decussation has been little studied (Manuel *et al.*, 2008). A critical question is to what extent loss of pigment leads to aberrant patterning of the CMZ and/or RPE and neural retina during optic cup development, and, “downstream”, the misrouting phenotype of the albino.

We identified one possible candidate patterning gene that could affect RGC development: In a microarray of albino vs pigmented embryonic RPE (Iwai-Takekoshi *et al.*, 2018), we found that Wnt genes were dysregulated in the albino RPE. Intriguingly, activating Wnt signaling through lithium treatment *in vivo* in pigmented mice during the period of RGC development led to a reduction in Zic2-positive cells, mimicking the albino phenotype (Iwai-Takekoshi *et al.*, 2018). Together, these data raise the possibility that activated Wnt signaling via the RPE underlies delayed neurogenesis/differentiation in the albino retina and in lithium-treated pigmented retina, affecting the competency of retinal progenitor cells to express Zic2, and consequently leading to a reduced number of ipsilateral RGCs. Future work must parse to what extent Wnts and other genes that pattern the RPE and are crucial to its development are upstream, downstream or act independent of melanin.

CLOSING REFLECTIONS

In our interchanges, Ray fretted that “...we are at a loss to know what the chain of events is in cause and effect” to produce the albino phenotype. He then added, “*Is there something we can learn from mouse studies on early development that will inform how the circuits are disrupted in cat?*” I argue that we have learned a good deal from the mouse studies: As discussed above, we know that the time of birth is linked to the expression of genes associated with laterality of projection, and that in the albino, neurogenesis and subsequently, cell identity, are altered. There is a lag in neurogenesis in the albino, and RGCs born at the close of neurogenesis tend to express contralateral genes; consequently, more RGCs in the albino VT retina project contralaterally rather than ipsilaterally. We know that molecular factors associated with patterning of the CMZ and RPE, such as Wnt 2b and Cyclin D2, are dysregulated in the albino retina. Finally, the integrity of albino RPE cells is perturbed. All of these developmental steps are necessary to act in sequence for retinal axons

to decussate in the proper ratio at the optic chiasm, to establish the binocular circuit (Figure 4).

To get to the bottom of the mechanisms underlying albinism, we will ask: What is the temporal relationship between melanin production, cell division and its control, and *Zic2* expression, especially in the CMZ, to regulate cell fate of ipsilateral RGCs? What are the signals deriving from the RPE that direct the proper tempo of neurogenesis and cell fate encoding RGC guidance through the chiasm and targeting in the thalamus and cortex? And what are the properties of RPE cells required to provide these signals? Armed with the knowledge we have gained from studying the altered circuitry of the albino visual system, in the name of Ray Guillery, I would like to spend the rest of my career unraveling this puzzle.

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Abbreviations

RGC	retinal ganglion cell
Ipsi	ipsilateral
contra	contralateral
dLGN	dorsal lateral geniculate nucleus
VT	ventrotemporal
DT	dorsotemporal
EM	electron microscopy
HRP	horseradish peroxidase
CMZ	ciliary margin zone

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Figure 1. Schema indicating how the visual field is represented in the lateral geniculate nucleus of normal (A) and Siamese (B) cats and in the visual cortex of Midwestern (C) and Boston Siamese (D) cats.

In the three small sketches, the arrows show the visual field positions pointing from lower to higher numbers, the asterisk* shows the position of the “hinge”, the gray line under the arrow represents few responses as do the pale numbers. See text for further details.

Figure 2. Morphology of mature (A, B) and immature (D) retinogeniculate axons labeled in vivo with horseradish peroxidase in cats and kittens (modified from (Mason & Robson, 1979);(Robson & Mason, 1979);(Mason, 1982b; Mason, 1982a). A, D: Camera lucida drawings. Mature axon arbors have large terminals with indented surfaces indicating sites of contact of dendrites and spines. Immature axons have growth cones and growing tips that have vesicles and make immature contacts. 9 D, 9 days. Numbered terminations in inset in C are numbered in the electron micrograph (for 3 only). d, dendrite; f, profile with pleomorphic vesicles. E, micrograph of immature growing tip (arrow, inset) containing synaptic vesicles, and synaptic densities along the length of the

growing tip. Scale bars: A, 25 microns; C, EM and inset, 2 microns; D, 10 microns; E, EM, 1 micron and inset, 4 microns.

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Transcription factors Guidance receptors

Figure 3. Distinct axon guidance receptors and ligands direct ipsi- and contralateral RGC divergence at the optic chiasm.

EphB1⁺ RGC growth cones spread and are repulsed by midline glia (not shown) expressing EphrinB2, and course ipsilaterally (green). Plexin-A1⁺ and Nr-CAM⁺ RGC growth cones interact with midline glia expressing Plexin-A1 and Nr-CAM as well as the inhibitory Sema6D and cross the midline (red). Not shown are: Sonic Hedgehog which is also repulsive to ipsilateral RGCs (Peng *et al.*, 2018) and VEGF which is attractive to contralateral RGCs (Erskine *et al.*, 2011). In addition, specific transcription factors (*italics*) control ipsilateral guidance programs (*Zic2*) and contralateral differentiation and guidance programs (*SoxCs*, *Islet2*, and *Brn3a*).

Figure 4. Schema of RPE-retinal precursor-differentiated RGC cell interactions in the pigmented retina and their perturbations in the albino retina.

These perturbations lead to a reduced ipsilateral projection and an increase in contralaterally-projecting RGCs. In comparison with the pigmented retina, 1. Albino RPE cells have fewer smaller gap junction particles and an irregular cell shape. In the absence of melanin or perturbed melanogenesis, factor “X” is missing or mutated. Factor X could regulate proper RPE cell biology (horizontal yellow and red arrows). 2. Factor X or a different factor is released (vertical yellow and red arrows) to affect neural retinal precursor proliferation in a timely manner, and could also affect Cyclin D2 (Ccd2) expression. 3. If timing of neurogenesis and/or Cyclin D2 is perturbed, then Zic2 expression is dysregulated (short vertical yellow and red arrows). Instead, RGCs are specified to be contralateral. 4. Fewer RGCs project ipsilaterally, and instead project contralaterally (dashed line) to the dLGN.