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Position along the nasal/temporal plane affects synaptic development by adult photoreceptors, revealed by micropatterning

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Abstract

In retinal degeneration, death of photoreceptors causes blindness. Repair of the retina by transplanting photoreceptors has resulted in limited functional connectivity between transplanted and host neurons. We hypothesize that absence of appropriate biological cues, specifically positional (retinotopographic) cues, reduces synaptogenesis. Here we use micropatterning to test whether regional origin affects the early synaptic development of photoreceptors. Right and left retinas from salamanders were first labelled with dextran tetramethyl-rhodamine and fluorescein, respectively, bisected into nasal (N)-temporal (T) or dorsal (D)-ventral (V) halves, individually dissociated, mixed, and cultured for 1 week. Origin of cells was identified by the fluorescent label. Interactions between photoreceptors and neighboring (target) cells were assessed by the number of neuritic contacts with a presynaptic swelling (varicosity). Randomly-plated photoreceptors showed no preference for cellular origin, likely due to multiple potential interactions available to each cell. To reduce cell-cell interactions, culture substrate was patterned using a microfluidic device with 10 μm -wide channels separated by 200 μm , thus allowing only 1–2 targets per photoreceptor. In patterned cultures, 36.89% of N rod cells contacted T targets but only 27.42% of N rod cells contacted N targets; similarly 35.05% of T rod cells contacted N cells but only 17.08% contacted T cells. Thus, opposite regions were more permissive of contact. However, neither cone nor D/V rod cells showed preferences for positional origin of targets. In conclusion, micropatterning demonstrated that neuritic differentiation by rod cells depends on

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retinotopographic cues along the nasal/temporal plane, suggesting that transplanting rod cells of known positional origin will increase transplant success.

Introduction

Transplantation of photoreceptors is currently seen as a potential palliative for many retinal diseases and injuries¹. We propose to test if there are positional preferences for the regenerative neuritic growth from photoreceptors which must occur after transplantation.

Similar to many other injuries and diseases of the central nervous system (CNS)², the degenerating retinal environment is less than optimal for integration. Integration is defined as the formation of the rod outer segment and the production of phototransduction proteins by the rod photoreceptor and is reported to be only 5–10%³. Additionally, high rates of synaptogenesis are critical for functional integration. Current research has shown that depending upon the disease model used for transplantation, the presence of synaptic proteins in “integrated” rod precursor cells varies widely from 30 to 60%³. Considering there are ~120 million rods and ~6 million cones in the human retina, replacing even a small fraction of these cells would require an enormous number of transplanted cells. However, increasing the number of photoreceptors injected into an area is not a viable strategy as this leads to progressively lower rates of integration and decreased thickness of the photoreceptor layer^{3, 4}. Therefore, increasing rates of integration and formation of synapses is key to the success of photoreceptor transplantation.

We hypothesize that transplanted photoreceptors may lack *synaptic* integration after transplantation due to the absence of appropriate retinotopographic or positional cues necessary to stimulate transplanted photoreceptors to form functional connections with the host tissue. Transplanted retinal tissue has long been known to exhibit retinotopographic specificity. In the classic experiment by Sperry et al. in 1943, retinal ganglion cells (RGCs) in the frog eye maintained topographic specificity with respect to the dorsal/ventral and nasal/temporal axes of the retina during synaptic reconnection to the host tectum even after transplantation into a new environment⁵. This experiment has been confirmed and repeated both in vitro and in vivo^{6–8}. Therefore, like RGCs, transplanted photoreceptors may display retinotopographic preferences after being placed into host tissue.

Retinotopography may not have been previously considered during photoreceptor transplantation because rod cells are not traditionally thought of as having retinotopographic specificity. However, several differentiation factors such as PAX6 and cVAX are differentially distributed across the surface of the retina and have been shown to have direct effects upon photoreceptors during development^{9, 10}. For instance, in an experiment by Schulte et al., rod photoreceptor patterns were disrupted by misexpression of the transcription pattern for cVAX⁹. Additionally, cone photoreceptors have long been known to form mosaic patterns across the retina and to be dependent upon their surroundings for proper differentiation^{11, 12}. The different types of bipolar cells that connect to photoreceptors are also unevenly distributed across the retina. For example, bipolar cell density is greater in temporal than in nasal portions of the primate retina¹³. Regional

differences in cell type densities and resulting positional cues, therefore, may contribute to the lack of integration of transplanted photoreceptors.

Photoreceptors are technically difficult to examine for retinotopographic cues due to the limited growth of their axons. Rod and cone cells have axons that are only tens of microns long compared to projection neurons such as dorsal root ganglion cells with axons that are hundreds of microns in length. Previous studies have shown that adult salamander rod cells do exhibit preferences towards new cell types (2nd vs 3rd order neurons) when cultured in well-controlled conditions by utilizing optical tweezers that ensure (i) a low number of potential targets per cell and (ii) specific intercellular distance¹⁴. Tweezing however is very time and resource consuming. In our studies, each animal yielded only ~ 8 cells suitable for analysis. Thus to obtain a large enough number of cells for statistical analysis many animals needed to be sacrificed. To combat these issues, we have developed a micropatterning-based method of culturing adult tiger salamander photoreceptors that increases the number of cells available for analysis while limiting the number of interactions each photoreceptor can have. This patterning technique was combined with an innovative staining procedure that makes it possible to identify nasal and temporal, or dorsal and ventral cells even after 1 week in vitro. Our approach yielded larger amounts of data on photoreceptor targeting than studies using optical tweezing.

This is the first study showing that rod photoreceptors are responsive to retinotopographic cues. Additionally, the custom cell patterning method used in this study demonstrates the utility of microfluidic devices for studying axon guidance and neural regeneration.

Materials and Methods

Animals

Retinal cells were obtained from adult, aquatic-phase tiger salamanders (*Ambystoma tigrinum*, 18–23 cm in length). The salamander culture system supports the survival and growth of adult retinal cells for long periods, unlike mammalian retinal cell systems, and cell type specific characteristics are retained^{15, 16}. In addition, the cultured salamander cells display neuritic sprouting characteristics reminiscent of human degenerative retinal diseases^{16–18}. Animals were maintained at 5°C on a 12-hour light/12-hour dark cycle for at least 1 week before use. All protocols were approved by the Institutional Animal Care and Use Committee at Rutgers Biomedical and Health Sciences and were in strict compliance with the RSC Publishing Experiments Involving Live Subjects statement.

Labelling of Salamander Retinal Cells

To obtain differentially labelled retinal cells, salamanders were enucleated and the anterior section of the eyeball except for the lens removed (Fig. 1). Using fine scissors, a small incision was made at the dorsal apex of the eyecup for reference. The vitreous was removed with a 10 µl pipette tip and replaced with 10 µl of salamander media (108 mM NaCl, 2.5 mM KCl, 2 mM HEPES, 1 mM NaHCO₃, 1.8 mM CaCl₂, 0.5 mM NaH₂PO₄, 1 mM NaHCO₃, 24 mM glucose, 0.5 mM MgCl₂, 1 mM Na pyruvate, 7% medium 199, 1× minimum essential (MEM) vitamin mix, 0.1× MEM essential amino acids, 0.1× MEM

nonessential amino acids, 2 mM glutamine, 2 µg/mL bovine insulin, 1 µg/mL transferrin, 5 mM taurine, 0.8 µg/mL thyroxine, 10 µg/mL gentamicin, and 1 mg/mL bovine serum albumin (BSA) (pH 7.7)) containing 10 mg/ml of either dextran fluorescein or dextran tetramethyl-rhodamine to left and right eyes respectively. Eyecups were then placed in a dark, humidified 10°C incubator. After 24 hours, the labelled retinas were bisected along either the nasal-temporal or dorsal-ventral axis using the incision as a reference. The retinas were then removed from the eyecup halves and dissociated as previously described¹⁹. Briefly, retinal halves were placed in 1.5 ml of papain solution consisting of 14 U/ml papain (Worthington, Freehold, NJ) in salamander Ringer's solution (85 mM NaCl, 1.5 mM KCl, 25 mM NaHCO₃, 0.5 mM CaCl₂, 0.5 mM NaH₂PO₄, 24 mM glucose, 0.03 mM phenol red, 1.0 mM Na pyruvate) containing 2.7 mM DL-cysteine for 40 minutes at room temperature. Each retinal half was separately rinsed twice in salamander Ringer's solution, and gently triturated using a wide-bore glass pipette. Solutions containing retinal neurons from each region of the eye were seeded onto unpatterned or patterned Sal-1 substrate as described below (Fig. 1). Cell density in suspensions of nasal-temporal or dorsal-ventral retinal cells were monitored to ensure equal plating densities for both groups. Cell densities were also examined after plating and found to be similar among all groups.

Microfluidic channel fabrication and patterning of the substrate, Sal-1 antibody

For unpatterned cultures, glass coverslips were coated with Sal-1, a hybridoma supernatant containing a monoclonal antibody raised against salamander retinal neurons, as previously described¹⁹. Sal-1 antibody allows for the attachment and growth of salamander retinal cells. To pattern the substrate, a microfluidic device was fabricated in poly(dimethylsiloxane) (PDMS) using standard soft lithography²⁰ (Fig. 2). The microfluidic channels were designed with an area that would comfortably fit onto a 25 mm no. 1 circular glass coverslip attached, using PDMS, to the bottom of a drilled 35 mm culture dish. An inlet and an outlet (1.5 mm wide by 11 mm long) were placed at the two ends of an array of 50 microchannels for introduction of culture substrate, the supernatant containing Sal-1 antibody (Fig. 2). The channels were 12 mm long and 10 µm wide. The width of the microchannels was chosen to match the typical diameter of cultured photoreceptors, which ensured that only one photoreceptor would be plated at a given location along the longitudinal axis of the microchannel. As a result, each photoreceptor had only 1–2 neighboring cells—one upstream and another downstream. The height of the microchannels was 50 µm in order to reduce their fluidic resistance and facilitate the flow of solutions during the patterning process. Microchannels were separated by a distance 20 times larger than the width of one channel, minimizing the interactions between cells on different “lanes” of Sal-1 patterned substrate.

After fabrication, the PDMS devices were briefly cleaned using Scotch® Transparent Tape 600 (3M, TwoHarbors, MN) and then covered in Scotch® Tape for storage until use. The prepared culture dishes were cleaned overnight in a 1% w/v Tergazyme (Alconox, White Plains, NY) solution, washed thoroughly in deionized (DI) H₂O, dried in a biosafety cabinet and sterilized by exposure to ultraviolet (UV) light for at least 1 hour. Cleaning the glass with Tergazyme solution and the PDMS devices with Scotch® Tape was essential in order to promote conformal sealing of the devices with the coverslips. The PDMS microfluidic

replica was reversibly sealed to the coverslip by gently pressing them together. The resulting channels were filled with goat anti-mouse IgG (0.1 mg/ml in salamander Ringer) and incubated for 3 hours at room temperature. After incubation, 50 μ l of salamander Ringer was washed through the channels, followed by the application of Sal-1 supernatant overnight at 10°C. The channels were then rinsed with 50 μ l of salamander Ringer and the microfluidic replica removed and discarded. Dishes were then filled with 2.5 ml of salamander media and seeded with combinations of nasal and temporal or ventral and dorsal labelled retinal cells. Cells were maintained in salamander media at 10°C in the dark. After 7 days, cells were fixed for 30 minutes with 4% paraformaldehyde in phosphate buffered saline (PBS), washed several times, and imaged with a Zeiss Axiovert 200M microscope (Carl Zeiss AG, Oberkochen, Germany).

Evaluation of contact formation between cells

As described in Fig. 3, photoreceptors were identified as cones or rods based on their morphology as previously described (cell shape, shape and size of the ellipsoid [a dense packing of mitochondria], and growth pattern)¹⁴. Researchers were blinded to the origin of cells being imaged and analyzed. For both unpatterned and patterned photoreceptors, every isolated photoreceptor within 70 μ m of another retinal cell was imaged (see Fig. 3); note that 70 μ m is the maximal length of neurites produced by these cells in vitro¹⁵. The contact of processes from the photoreceptor to other cell types was evaluated and the formation of presynaptic varicosities (defined as swellings of at least 0.5 μ m in diameter along neurites) was noted. Data were analyzed using the Chi Squared test for independence. Data were considered significant at $p < 0.05$.

Results

Labelling retinal cells

By using fluorescent dextrans, we successfully labelled all retinal cell types in all retinal regions. Dextran enters cells via endocytosis²¹ which can be seen in retinal cell culture as labelling of the inner segments of photoreceptors or as labelling of areas of high endosomal content in the cytoplasm of other retinal neurons. After seven days in culture, the fluorescent dextrans remained in the retinal cells allowing us to visualize the regional origin of each photoreceptor cell as well as the second and third order retinal neurons (bipolar, horizontal, amacrine and ganglion cells). Retinal cells did not take up significant quantities of dye in the culture medium potentially leftover from the dissociation based upon the lack of “double” labelling in retinal cells. Additionally, label density did not appear to affect cell survival. For our experiments, we only analyzed cells with clear fluorescent labelling.

To test if the dye increased varicosity formation in nasal or temporal cells preferentially, we compared the interaction between nasal cells from the left eye and nasal cells from the right eye with the interaction between temporal cells from the right and left eyes. No difference in varicosity formation was seen between either all nasal or all temporal cell cultures (data not shown). Comparison of growth from right and left eye-derived cells in the all nasal or all temporal cultures indicated that there were no differences in growth between cells from the right versus the left eye (data not shown).

Photoreceptor targeting on unpatterned culture dishes

From previous work we know that photoreceptors form numerous actin-containing sprouts from all regions of the perimeter of the cell after 1–2 days in culture. Some of these sprouts develop further and contain microtubules, at which point they can be called neurites. The neurites are able to form large swellings called varicosities which are filled with vesicles containing presynaptic proteins such as SV2 and synaptophysin¹⁶ and contain voltage-gated calcium channels on the plasmalemma²². The number of neurites and varicosities does not increase significantly after about 1 week in culture, although the varicosities do begin to be synaptically active^{15, 16, 23}. In the current work, label density did not appear to affect the differentiation of neurites or varicosities. Moreover, fluorescent dextrans could occasionally be found in large varicosities indicating vesicular transport of the dye to these areas of the cell (see Fig. 4A).

Previous work has shown that neuritic processes with presynaptic varicosities preferentially grow either towards or away from specific cell types^{14, 23}. In this study, any cells present within 70 μm of the center of a selected photoreceptor were considered target cells for that photoreceptor. Target cells were grouped based upon their regional origin, i.e. nasal or temporal cells, and further categorized based upon the presence or absence of a contact from the selected photoreceptor and whether or not the contacting process contained a varicosity. The importance of looking at processes with varicosities is that these swellings are evidence of axonal differentiation and are the first signs of synaptogenesis. No attempt was made to determine the cell type of the target cell (i.e. bipolar, amacrine, or ganglion) because, after several days in culture, only photoreceptors can still be reliably identified based on their morphology. Under the conditions of random (unpatterned) plating, and in contrast to expectations based on previous work, rod and cone photoreceptors did not demonstrate any significant preference for or avoidance of cells from other regions of the eye (Fig. 4). However, each photoreceptor had an arbitrary number of available targets, which made analyses subject to much variability. In order to rectify this issue, we devised a method of patterning retinal neurons to control the number of potential interactions available to each photoreceptor.

Photoreceptor targeting on patterned culture dishes

To address the number of potential targets that each cell can interact with, we utilized an array of microfluidic channels to pattern the Sal-1 substrate, to which retinal neurons attach. For visualization of the patterns, Sal-1 was stained using a fluorescent G α M antibody (Fig. 2C). Photoreceptors grown on Sal-1 patterns generally remain within the boundaries of the Sal-1 pattern; only some thinner sprouts were able to extend past the borders of the Sal-1 pattern over time (Fig. 2D). Any cells that attached to areas outside of the Sal-1 pattern, which happened infrequently (Fig. 2E), were not included for analysis and any photoreceptor nearby such a cell was not analyzed. These Sal-1 patterns allowed us to reduce the number of potential interactions each photoreceptor could make. As shown in Fig. 5A, each cell was limited to interactions with only 1 or 2 other cells. The placement of cells on the pattern was random.

We first examined cone cell growth in response to the presence of target cells of similar or different regions of the eye. Cone cells on Sal-1 stripes grew similarly to cones grown on unpatterned Sal-1 and tended to form 3–4 neurites from anywhere along the perimeter of the cell. Varicosities formed on these neurites. Nasal and temporal cones did not show any preference for nasal or temporal targets in either neuritic contact or the formation of varicosities. Dorsal and ventral cones also did not demonstrate any preference for dorsal or ventral targets in either neuritic contact or the formation of varicosities (Fig. 5B).

We then examined rod responses to potential retinotopographic cues. Rod cells on Sal-1 stripes grew similarly to rod cells grown on unpatterned Sal-1 substrates and had many more thin processes than cone cells. The thin processes tended to be linear without any change in direction from their initial sprout and were able to extend past the boundaries of the Sal-1 stripes. Thick rod neurites, however, remained restricted to the Sal-1 substrate boundaries similarly to the neurites of cone cells. When we looked at nasal and temporal rods, we found that although there were no preferences in contact formation with other nasal and temporal targets when all processes were considered, preferences did appear when varicosity-containing neurites were analyzed. Nasal rod cells formed varicosity-containing neuritic contacts with temporal targets in 36.89% of the cases and with nasal targets in only 27.42% of the cases. Similarly, temporal rods formed varicosity-containing contacts with nasal targets in 35.05% of the cases and with temporal targets in only 17.80% of the cases. (Fig. 6B). Thus regional preferences were found for rod cells with differentiating axons as evidenced by the presence of presynaptic varicosities.

In contrast to results along the nasal-temporal axis, dorsal and ventral rods did not exhibit any preference for dorsal or ventral targets in both number of contacts or the formation of varicosities along neuritic contacts (Fig. 6C).

Distance between photoreceptor and target cell, a possible variable, did not significantly affect neuritic or varicosity formation in either rod or cone cells. In rod cells, the standard deviation of percent of neuritic contacts and varicosity contacts was only 3.8% and 2.6% respectively. For cone cells, the standard deviation of percent of neuritic contacts and varicosity contacts was 6.3% and 8.4% respectively (Supp. Fig 1). Thus for our experimental conditions, neither the number of contacts nor the formation of varicosities along neuritic contacts were affected significantly by variations in the distance between photoreceptors and target cells.

In contrast to distance, patterning did affect the frequency of contact and varicosity formation in comparison to unpatterned rod and cone cells. In this study, rod cells formed contacts with neighboring cells 15.18% more often on patterned substrates than on unpatterned substrates ($p < 0.0001$). Similarly, rod cells on patterned substrates formed varicosity-presenting contacts with neighboring cells 17.02% more often than on unpatterned substrates ($p < 0.0001$). Cone cells on patterned substrates formed contacts with neighboring cells 19.39% and varicosities 22.04% more often than on unpatterned substrates ($p < 0.0001$) (Fig. 7).

Discussion

In this study, we examined the effect of regional origin of target retinal cells on photoreceptor sprouting and early synaptic development. We showed that by utilizing micropatterning to reduce the confounding variables in a culture system, one can glean new information about factors that affect the regeneration of photoreceptors. Our experiments demonstrate that rod cells prefer to form presynaptic varicosities when in contact with cells from the opposing region in the nasal-temporal plane of the eye. Cone cells on the other hand, do not seem to exhibit any preference in target cell positional origin. Our findings follow a trend seen in previous work¹⁴: that rod photoreceptors seem to prefer “novel” instead of “normal” targets—in this case, “normal” targets are those from the same nasal or temporal area of the eye as the contacting photoreceptor.

An important feature of our micropatterning approach is that it enabled high-throughput studies which generated large amounts of data on axon guidance while requiring minimal time and resources. In general, patterning cells along single-cell lanes allows researchers to quickly examine interactions between pairs of cells that are separated from other pairs. In this study, micropatterning made it possible for us to examine 3579 cells using only 9 animals in comparison to optical tweezing or random plating, which examined only 203 or 482 cells and required 55 or 24 animals respectively. Micropatterning is also simpler, less expensive, and more accessible to untrained researchers than other cell-positioning methods such as optical tweezing or cell printing. This micropatterning method could potentially be used to study the interactions between other cell types. For example, in research examining glial and neural interactions, multiple neural and glial contacts can make it difficult to ascertain the nature of their interaction. The micropatterning method presented here could allow researchers to examine the interactions between a single neuron and a single glial cell. A similar technique has already been employed for studying cell-cell contacts in primary cardiac myocytes²⁴. In addition, this technique increases the frequency at which relatively rare events occur and therefore allows researchers to gather data more quickly about such events. For example, in order to acquire the same number of total varicosity contacts in unpatterned cultures as those acquired in patterned cultures, we would have needed to examine approximately 3 times more rod cells and approximately 3 times more cone cells (Fig. 7). The increase in the number of contacts observed in patterned cultures most likely arises from the fact that while neurites continue to sprout from all areas of the cell body, they preferentially grow on the patterned Sal-1 substrate, which limits the direction of growth and increases growth towards and contact with target cells. We have data demonstrating that contact increases the number of varicosities in both rod and cone cells with contact-mediated stimulation of varicosity formation appearing more pronounced in cone than rod cells¹⁴. In our patterned cultures, cone cells also increased varicosity formation slightly more than rod cells. Thus, the substrate pattern increased cell-to-cell contact and this contact in turn likely increased production of varicosities.

The micropatterning method presented here, however, could be further optimized in several ways depending on experimental requirements: (i) By controlling the distance between cells - variability in the distance between cells did not play a major role in this study, (Supp. Fig. 1) because we were able to choose pairs separated by a predetermined maximum distance

and still have adequate numbers of cell pairs for statistical significance. (ii) By selecting the exact two types of cells present in each pair. —in our study we solved this possible limitation by having adequately high numbers of all types of cell pairs (N-N, N-T, T-T, etc.) for statistical significance; in the future, selecting for nerve cell type (bipolar, amacrine, ganglion, etc.) in addition to retinal region could determine if there is cell specificity within retinal regions. And (iii) by controlling cell migration off the patterned substrate. In our cultures, retinal cells do not migrate. For other cell types however, migration might be a concern; it can be reduced by using BSA, poly(HEMA), Teflon, or other cell-repelling substrates between the patterned stripes. We have successfully applied this approach to fibroblast cultures on a patterned substrate²⁵.

Our results have implications for increased efficacy in retinal transplantation. Rod cell transplantation to replace lost photoreceptors is a potential therapy for retinitis pigmentosa (RP) and other retinal degenerative diseases. However, studies so far have resulted in only mild success and much variability in transplantation efficiency with integration rates ranging from 5 to 10%³. Functional recovery in these studies has varied from pupillary light responses to behavioral responses to visual stimuli^{1, 26}. One possibility for this lack of both repeatability and integration is that transplantation studies are typically unconcerned with the area from which they obtain the transplant or where the replacement rod photoreceptors are eventually transplanted. This could lead to incongruous guidance signaling preventing proper synapse formation between the transplanted photoreceptors and the host tissue, regardless of the developmental stage of the transplanted cell (whether adult or precursor cells). Based on our results, it seems that inhibitory factors to varicosity formation exist between rod photoreceptors and other retinal cells from similar areas of the eye. Counterintuitively, it may be more beneficial to transplant rod cells from opposite rather than similar areas of the eye. To improve efficacy, researchers could examine which cell populations, based on retinal origin, have the most success in properly integrating with the diseased retina. By injecting only nasal or temporal cells into the retina, for instance, they could determine i) if one population has higher integration rates and therefore better visual recovery than mixed transplants and ii) what region of the retina is most receptive to the transplant. Although photoreceptor to bipolar interactions would be optimal, conceivably any synaptic interaction that conveys light sensitivity to the retina would be of therapeutic value. Alternatively, it may be necessary to use photoreceptors modified to express specific levels of guidance cue receptors in order to increase their ability to correctly synapse onto their proper bipolar cell partners. Modifications of responses to guidance cues have already been examined in other transplantation models and found to increase integration^{19, 20}. Our study documents that a regionally specific cue may be involved in this process.

Although significant differences in contact and varicosity formation between nasal or temporal rod cells and temporal or nasal target cells, occurred, no differences in contact and varicosity formation appeared between the dorsal and ventral photoreceptors and targets. In humans, the differences in cell types and densities are more pronounced in the nasal-temporal in comparison to the dorsal-ventral regions¹². RP is also known to start peripherally where rod cell densities are highest²⁷ although it is unknown if rod neuritic sprouting seen in RP is affected by regional location. Additionally, the guidance cues or transcription factors affecting the formation of varicosities may be present only in the nasal-

temporal and not the dorsal-ventral plane. In fact, several guidance molecules and transcription factors that control the expression of these guidance cues, including Eph/Ephrins^{28–30}, BF-2³¹, MEK-4³², and Foxd1³³, display a nasal-temporal but not a dorsal-ventral pattern of expression. The Eph-Ephrin family of guidance molecules may be particularly interesting in this regard as the Eph-Ephrin signaling pathway seems to play a role in synapse formation²⁸. However, making simple assumptions about guidance cue gradients based on normal eyes may be deceptive as studies have shown that injury can cause changes in the gradient of these molecules in the retina³⁴.

In contrast to rod cells, cone cell growth in our system showed no regional preferences. There are several types of cone cells in salamander, as well as mammalian retina^{35, 36}. Perhaps individual cone cell types do have preferences which are masked when all cone cell types are combined for analysis. In human retinal degeneration, cone cells appear less reactive in terms of injury-induced neuritic growth^{17, 18} and thus, alternatively, cone cells may have few active signaling systems for axonal guidance.

Our results may have implications for other cell types in the retina as well. For example, bipolar, horizontal, and ganglion cells also undergo many morphological changes in the retina following retinal degenerations or injuries³⁷. How these cells respond to changes in guidance cues after injury is unknown despite recent discoveries of some of the guidance molecules which help to form the inner synaptic laminae of the retina^{38, 39}. In the future, creating stripes of guiding molecules known to play a role in the developing retina in combination with markers for second and third order neurons (such as PKC α , calbindin, and Thy-1) in adult salamander cultures of specific retinal halves (e.g. nasal, temporal, dorsal, or ventral) may provide new information on regenerative growth and synaptogenesis after injury.

Finally, our work also suggests that neuritic growth and differentiation—demonstrated by the formation of thick processes and presynaptic varicosities—of rod cells are controlled by separate mechanisms. This study shows that contact between the photoreceptor and its target cell did not vary depending on the origin of the target cell. Varicosity formation, however, was affected by the target's regional origin. Therefore, the growth of neuritic processes may be controlled in a different manner than the formation of varicosities. This independence between process and varicosity/synapse formation has been demonstrated in other neuronal cell types in both the CNS and the PNS. For example, in the hippocampus, dendritic morphology can be controlled by synaptic activity³⁰, while spine morphology is controlled by the ephrin-A3/EphA4 system⁴⁰. In the PNS, limb motor axons guidance can be controlled by EphA4 tyrosine kinase activity⁴¹, while innervation of muscles is controlled by muscle specific tyrosine kinase (MUSK) along with other factors.

In sum, our relatively straightforward use of micropatterning has revealed new specificities in the connectivity preferences of photoreceptors. We expect the uncovered retinal preferences to have a significant impact on the therapeutic efforts towards regenerative repair in the retina. Additionally, as the visual system is often a model for the larger CNS, our findings and methodology should be useful in the study of other neural systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Insight Box

This article demonstrates that isolated photoreceptors, similar to preparations used for retinal transplantation, are sensitive to retinotopographic cues. Specifically, we found that presynaptic formation by adult rod photoreceptors is modulated by positional information along the nasal/temporal axis. This finding depended on the use of a microfluidic device to pattern the retinal-specific substrate. Without patterning, each photoreceptor in culture can potentially contact many targets. By substrate patterning, we reduced the number of potential interactions between photoreceptors and target cells to only 1 or 2. Thus we have shown that by integrating micropatterning with retinal cultures, researchers can reduce the number of environmental variables in vitro and discover new phenomena governing neuronal growth and repair of retinal tissue.

Fig. 1.

Cell preparation. Sequence of steps involved in harvesting, differentially labelling, and culturing of retinal cells on unpatterned or patterned substrates. 1–3. After enucleation and removal of the anterior segment, a fluorescent dextran dye was added to each eyecup and the eyecups were incubated for 24 hrs. 4–6. Each eyecup was bisected along the nasal-temporal or dorsal-ventral axis. Individual retinal halves were removed and dissociated into cell suspensions separately. 7) Cell suspensions from opposing eyes were mixed and plated on either unpatterned or patterned substrates for 7 days. T, temporal, N, nasal, D, dorsal, V, ventral.

Fig. 2. Cell micropatterning. A) Sequence of steps required to pattern the substrate, Sal-1, used for retinal cell culture. Photoresist, a UV sensitive polymer. PDMS, a silicon polymer that forms a flexible relief of the photoresist structure. B) Detailed view of the mask. C) Fluorescence image of a single Sal-1 stripe on glass. Secondary antibody was added to visualize the Sal-1 antibody, which is not fluorescent. Scale bar = 10 μm . D) Phase-contrast image of retinal cells plated onto a patterned Sal-1 substrate. Scale bar = 50 μm . E) Fluorescence image of rod photoreceptor cultured on a patterned substrate for 7 days. White dotted lines indicate the borders of the Sal-1 pattern (not immunolabelled here). Cell is immunolabelled for rhodopsin, a rod specific protein. Scale bar = 10 μm .

Fig. 3.

Analysis of the interactions between photoreceptors. Sequence of steps taken to quantify the interaction between photoreceptors and their targets. Rod and cone cells were identified by morphology. Target cells were identified as any cell within a circle with a radius of 70 μm around the identified photoreceptor. Interactions were categorized in three ways: no contact; contact by a process from the photoreceptor onto the target cell, a neurite contact; or presence of a presynaptic varicosity on the contacting process, a varicosity contact. N, nasal, T, temporal, D, dorsal, V, ventral.

Fig. 4. Interactions of cone and rod photoreceptors with target cells on unpatterned substrate. A) Green (i.e., from the left eye) salamander cone cell after 7 days in culture. Dashed line displays the 70-µm radius circle within which possible targets for the cone cell were considered. Arrowheads indicate varicosities. B) Percentage of cone cells making contacts with target cells—either right-eye (red) temporal cells contacting left-eye (green) nasal cells or right-eye (red) nasal cells contacting left-eye (green) temporal cells. N = 943 cells. C) Red (i.e., from the right eye) salamander rod cell forming contacts with another cell from the same region of the eye. Arrowhead indicates a varicosity. D) Percentage of rod cells making contact with target cells—either right-eye (red) temporal cells contacting left-eye (green) nasal cells or right-eye (red) nasal cells contacting left-eye (green) temporal cells. N = 809 cells. No statistical differences between groups were seen. Images were processed for clarity.

Fig. 5. Cone cells on patterned substrate. A) Red (i.e., from the right eye) temporal cone cell contacting cells from both areas of the eye: right-eye temporal cell (red) and left-eye nasal cells (green) after 7 days in culture. Arrowheads indicate varicosities containing fluorescent dextran. B) Percentage of cone cells from nasal/temporal regions growing on patterned substrates making contacts with target cells—either right-eye (red) temporal cells contacting left-eye (green) nasal cells or right-eye (red) nasal cells contacting left-eye (green) temporal cells. N = 849 cells. C) Percentage of cone cells from dorsal/ventral regions on patterned substrate making contacts with target cells—either right-eye (red) dorsal cells contacting left-eye (green) ventral cells or right-eye (red) ventral cells contacting left-eye (green) dorsal cells. N = 1274 cells. No statistical differences between groups were seen for neuritic contacts either with or without varicosities.

Fig. 6.

Rod cells on patterned substrate. A) Example of varicosity formation by a red (i.e., right-eye) rod cell with a target cell from the opposite area of the other eye (i.e., green, left eye). Arrowheads indicate varicosities. B) Percentage of rod cells from nasal/temporal regions growing on patterned substrates making contacts with target cells—either right-eye (red) temporal cells contacting left-eye (green) nasal cells or right-eye (red) nasal cells contacting left-eye (green) temporal cells. The total number of contacts—i.e., neurite and varicosity contacts combined—remained similar for all types of cell-cell interactions. Nasal and temporal rod cells, however, did show significant preference for forming varicosity contacts with target cells from a different area of the eye—i.e. nasal rod cells contacted significantly more temporal targets and temporal rod cells contacted significantly more nasal targets when looking only at the varicosity contacts. $N = 702$ cells. * $p < 0.05$, *** $p < 0.0001$. C) Percentage of rod cells from dorsal/ventral regions on patterned substrate making contacts with target cells—either right-eye (red) dorsal cells contacting left-eye (green) ventral cells or right-eye (red) ventral cells contacting left-eye (green) dorsal cells. No statistical differences between groups were seen. $N = 754$ cells.

Fig. 7.

Cone and rod contacts on unpatterned versus patterned substrates. Percentage of neurite and varicosity contacts between rods or cones and target cells. Patterned substrates produced significantly more varicosity contacts which resulted in significantly higher numbers of total, neurite and varicosity, contacts (* $p < 0.0001$). $N = 803$ unpatterned and 1456 patterned rod photoreceptors and 954 unpatterned and 2123 patterned cone photoreceptors.